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Synthetic Dna Technology As A Tool To Generate Vaccine Immunity In The Skin

Abstract

Since DNA's ability to generate an immune response was first described over 25 years ago, much work has been done to realize DNA's full potential as a safe and potent vaccine candidate. Renewed research has focused on continually improving the potency of the platform, which has led to advancements in electroporation, DNA formulation, and novel synthesized sequence optimizations, allowing newer "synthetic" DNA vaccines (SDNA) to contend as a major vaccine platform. Further insights into factors that influence SDNA vaccine outcomes are critical to achieving full potential. Here, we designed novel SDNA encoded skin-derived cytokines within the IL-36 family, to assess their impact on immunity against several viral targets. Zika virus challenge studies were also performed to assess whether observed adjuvant activity led to improved challenge outcome. The studies show that codelivery of optimized IL-36 gamma, with a non-protective dose of a Zika SDNA vaccine, can enhance immune responses, allowing for protection against challenge compared to nonadjuvanted mice. Another important area that is relatively understudied is skin delivery of SDNA vaccines. The skin is a major immune organ, and expanded applications for immunization might be possible with better understanding of its potential in the context of newer SDNA technology. To test the impact of skin vaccination on a relevant pathogen challenge, two consensus SDNA vaccines that encode a Leishmania antigen, PEPCK, were designed incorporating several genetic improvements including RNA and codon optimization and addition of a highly efficient IgE leader sequence. These were used to immunize mice intramuscularly or intradermally and analyze the resulting immunity. We observed that intradermal vaccination drove a greater number of antigen specific skin resident T cells in the skin compared to intramuscular vaccination, both at the vaccination and distal site. We further observed that mice immunized intradermally were better protected against parasite challenge and burden compared to intramuscularly immunized mice. My thesis supports the idea that the skin represents both a robust source of important immune modulators that can improve vaccination outcome and a unique site for SDNA immunization that gives rise to long lived resident immune cells which may play a crucial role in generating effective interventions against infectious agents and cancer.

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SYNTHETIC DNA TECHNOLOGY AS A TOOL TO GENERATE VACCINE IMMUNITY IN THE SKIN

Lumena Louis

A DISSERTATION

in

Pharmacology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2019

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Dedication

I dedicate this dissertation and the entirety of my Ph.D. to my mother. In spite of the challenges, you have always kept me grounded and reminded me of who I am. This isn't the doctorate degree that you hoped for me, but I hope that I have still made you proud. I can only pray that this degree will allow me to take care of you and give you the life that you deserve. I could not have asked for a better mother. I love you.

ACKNOWLEDGMENT

I first acknowledge God, for with Him, all things are possible. You've kept and sustained me on this journey and guided my footsteps. You have watched over me through a number of valleys and dark places, and it is throughout this Ph.D. that I have glimpsed some of Your mercy and grace. I will forever carry ***Psalm 27*** with me.

I came into graduate school thinking that my previous lab experiences and postbaccalauerate experience had prepared me for what was to come. Of course, I was in for a terrible shock. I would like to thank my thesis advisor, Dr. David B. Weiner, who took me into his lab and saw in me what other PIs may have missed. While I'm sure a lot of it has to do with my Brooklyn charm, Dave has pushed me to be the best scientist I can be at this stage, despite and in spite of my ***strong*** aversion to mouse work. Dave has always insisted on preparing his students for life after graduate school and his lab, and I am thankful for his constant charge and challenge for me to grow and evolve out of habits that may not serve me in the future. I have long admired Dave's ability to analyze situations from multiple angles before taking action, as well as his willingness to have tough conversations even when they force him to reevaluate his own position and thinking. Dave's ability to laugh and make terrible dad jokes even in stressful situations continues to amaze me, and reminds me to not always take myself so seriously. Lastly, I hope that I will finally get a chance to watch the many movies that Dave has recommended to me over the years.

I'd like to thank Dr. Kar Muthumani for his advice, especially in the later experiments of my thesis work with the Zika studies. I would be remiss if I did not thank Dr. Philip Scott and his lab at the Veterinary School at the University of Pennsylvania for our joint collaboration on the Leishmania skin vaccine studies.

I want to thank my thesis committee members, Dr. Rebecca G. Wells, Dr. Vladimir Muzykantov, Dr. Ronald G. Collman, and Dr. Nicola J. Mason for their guidance throughout my Ph.D. The wide breadth of expertise of my committee has led to several interesting conversations that have forced me to examine my work from a number of different angles.

A major thank you to the Ernest E Just (EE Just) Biomedical Society; the society has been a source of comfort throughout the struggles, both common and unique to us, of graduate school. I will always cherish the fellowship we have shared throughout the years, and carry these friendships with me. I would also like to thank the PennPREP program for the structure and extra lab experience that I needed prior to graduate school. I would like to specifically thank Dr. Arnaldo Diaz and Dr. Jean Boyer who served as co-chairs of the program while I was a member.

I would also like to thank my fellow classmates in the Pharmacology graduate group. Thanks for your patience in trying to get a homebody like myself to come out and have some fun! I tried my best, but I was always better at happy hour and dinner, than I was at the late night bar crawls!

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Most importantly, I would like to thank my family who has been with me from the very beginning. Mommy, you've been my Day 1! You believed in me even when I did not believe in myself,

reminded me that all things are temporary, and that God has brought me too far to let me go now. I love you always! Cheyenne and Whislyn, you have to call me doctor for a week! But on a more serious note, I am inspired and in awe of the fine young women that you both have become. I've watched as you have flourished from students into career professionals. Of the many things that I've learned from you, perhaps the most important has been the absolute importance of pursuing a path that will bring you happiness; that so long as you can look at yourself in the mirror and be content with the road you've traveled, the struggle will have been worth it.

ABSTRACT

SYNTHETIC DNA TECHNOLOGY AS A TOOL TO GENERATE VACCINE IMMUNITY IN THE SKIN

Lumena Louis

David B. Weiner

Since DNA's ability to generate an immune response was first described over 25 years ago, much work has been done to realize DNA's full potential as a safe and potent vaccine candidate. Renewed research has focused on continually improving the potency of the platform, which has led to advancements in electroporation, DNA formulation, and novel synthesized sequence optimizations, allowing newer "synthetic" DNA vaccines (SDNA) to contend as a major vaccine platform. Further insights into factors that influence SDNA vaccine outcomes are critical to achieving full potential. Here, we designed novel SDNA encoded skin-derived cytokines within the IL-36 family, to assess their impact on immunity against several viral targets. Zika virus challenge studies were also performed to assess whether observed adjuvant activity led to improved challenge outcome. The studies show that codelivery of optimized IL-36 gamma, with a non-protective dose of a Zika SDNA vaccine, can enhance immune responses, allowing for protection against challenge compared to nonadjuvanted mice. Another important area that is relatively understudied is skin delivery of SDNA vaccines. The skin is a major immune organ, and expanded applications for immunization might be possible with better understanding of its potential in the context of newer SDNA technology. To test the impact of skin vaccination on a relevant pathogen challenge, two consensus SDNA vaccines that encode a Leishmania antigen, PEPCK, were designed incorporating several genetic improvements including RNA and codon optimization and addition of a highly efficient IgE leader sequence. These were used to immunize mice intramuscularly or intradermally and analyze the resulting immunity. We observed that

intradermal vaccination drove a greater number of antigen specific skin resident T cells in the skin compared to intramuscular vaccination, both at the vaccination and distal site. We further observed that mice immunized intradermally were better protected against parasite challenge and burden compared to intramuscularly immunized mice. My thesis supports the idea that the skin represents both a robust source of important immune modulators that can improve vaccination outcome and a unique site for SDNA immunization that gives rise to long lived resident immune cells which may play a crucial role in generating effective interventions against infectious agents and cancer.

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" And most importantly, I know that we need to directly teach our children the most vital lessons, rather than assume that they'll be understood." Galit Breen

Scene: Lumena, age 5 having a full-blown meltdown at having to take the TDp/dTp vaccine

"I DON'T CARE that it's good for me. I hate needles, and I don't want to take this shot!!!!" (tears streaming down young Lumena's face)

"It will be over before you know it. Just a little pinch and that's it!" (exasperated nurse who just wants to get on with her day).

"Don't cry, don't be scared. You need this shot. You don't want to get sick if something happens, do you? This will help prevent that. We'll go to Mcdonald's afterwards." (Lumena's mom trying to bribe and distract her from this scary needle about to go in her arm).

"Can I get some help in here?" (nurse calling for coworkers to help her and mom hold down Lumena's limbs to administer the shot)

CHAPTER 1- Introduction

The skin as a protective barrier and immune organ

Early vaccination efforts can be traced back to the practice of variolation during the 16th century in China, as a means of protection against the debilitating and oftentimes scarring and deadly effects of smallpox (Boylston 2012). In some of the earliest records documented, accounts of people inhaling dried and crushed smallpox scabs from the skin of an infected person to protect against severe disease show that even early on, there was some understanding of pathogen exposure and resulting immunity. It was not until the 18th century when Edward Jenner, a physician scientist, remarked early in his life that milkmaids who were exposed to cowpox appeared immune to smallpox, created the first successful vaccine against smallpox that the observed protection of inoculated cowpox against smallpox was fully realized (Plotkin 2014). Smallpox remains the only human disease fully globally eradicated as of today.

The modern day smallpox vaccine is made of live vaccinia virus and is delivered to the skin of the arm by puncturing the skin with a bifurcated needle 15 times (Belongia and Naleway 2003). Precaution must be taken to prevent the live virus from spreading from the site of vaccination, however very serious adverse complications from vaccination are relatively rare. The smallpox vaccine has historically had an efficacy rate of 95%, with life long immunity, which was commonly attributed to the immunogenicity of the virus. However, a study comparing the delivery of the smallpox vaccine in the skin to delivery in the muscle has shown that given the same dose, intradermally vaccinated mice are better protected against smallpox challenge compared to intramuscularly immunized mice (Liu et al. 2010). The same result was observed in a melanoma model, supporting the ability of the skin to play a major role in facilitating a robust, protective immune response enhancing the immunogenicity of the delivered vaccine antigen.

The skin is the largest organ of the human body and plays a major role in maintaining several homeostatic processes in the host (Gruber and Schmuth 2014). These processes include

waterproofing, limiting dehydration, providing strength and flexibility, regulating pigmentation, and maintaining insulation. Skin is made up of three physical layers: the epidermis, the dermis, and subcutaneous layer (Losquadro 2017). In addition to providing a physical barrier, the skin also acts as a first line immune defense through which the innate and adaptive arms can be activated. This occurs by way of a number of cell types that reside in the skin, including keratinocytes, melanocytes, $CD4^+$ and $CD8^+$ T cells, Langerhans cells, dendritic cells, macrophages, natural killer cells, innate lymphoid cells and fibroblasts (Abdallah, Mijouin, and Pichon 2017).

Keratinocytes, which make up approximately 95% of the skin are primarily thought to ensure the skin's structural integrity (Eckert 1989). In addition to their physical function, keratinocytes are often considered immune guard keepers, for their ability to sense a number of external stimuli and in response secrete a number of cytokines, chemokines, and antimicrobial peptides (Nagy and Kemény 2009). These molecules can either recruit other immune cells or directly destroy the offending material. Molecules in this category include IL-1, IL-10, TNF-α, TGF-β, IL-6, G-CSF, IP-10, CXCL-8, cathelicidins and β-defensins and possibly others (Hesse-Macabata et al. 2019). Keratinocytes can also engage in cross talk with dendritic and T cells, further engaging the adaptive response and initiating a cascade to facilitate immune memory.

Langerhans cells (LCs), members of the dendritic/macrophage family, are considered the major antigen-presenting cell located in the epidermis (Mutyambizi, Berger, and Edelson 2009). They play a major role in immune tolerance during normal physiologic conditions, and a critical role in initiating inflammatory events when danger is present. LCs extend their dendritic processes between intercellular tight junctions to sample the environment in the uppermost regions of the skin, and are likely the first cells that skin-invading pathogens encounter (Deckers, Hammad, and Hoste 2018). It has been established that LCs express major histocompatibility complex II (MHC II) and can therefore present antigens to $CD4^+$ T cells. There is some conflicting data in the field whether true LCs express MHC I and cross present to $CD8^+$ T cells, however it is evident that

LCs play a major role in initiating innate and adaptive immune responses. LCs can capture and process antigens through the pattern recognition receptor (PRR) Langerins (Clayton et al. 2017), and then migrate to local draining lymph nodes where they can interact with naïve T and B cells in order to initiate the adaptive immune response.

T cells found in the skin are usually either resident or effector T cells that are antigen experienced (Clark 2015). After the initial proliferation of a naïve T cell that has been activated by an APC, a contraction in the number of T cells specific for an antigen occurs, however T cells can persist at numbers higher than before antigen encounter even after infection clearance. Data support that the T cells that remain help maintain memory against the pathogen, so that the immune response mounted upon a second encounter will be more rapid and of greater magnitude. A subset of these T cells will remain effector memory T cells (T_{EM}), where they travel around the body outside of the lymphoid tissues fighting off secondary infections (Gray, Westerhof, and MacLeod 2018). Exciting work in the T cell field has begun to identify distinct subpopulations of T effector cells, including $Tcf1^{+/-}PD\text{-}1^{+}$ cells, which appear to be critical for stem-like renewal of effector cells, especially in the context of cancer, tumor burden control, and T cell exhaustion (Siddiqui et al. 2019). Another subset of T cells will become resident memory T cells (T_{RM}), where they are thought to remain indefinitely in the tissue and do not recirculate (Carbone 2015). These T cells fight off infection directly in the tissue and are considered to be the first line defense against pathogen reencounter. T_{RM} are generally found in epithelial tissues where there is constant exposure to the outside environment, including the skin, gut, and lungs (Shin and Iwasaki 2013).

DNA vaccine technology and the skin

Vaccines are considered among the top accomplishments of modern medicine, saving millions of lives by inducing immunity to a number of infectious pathogens. As the next generation of vaccines seeks to address ever more complicated targets including cancer, innovative technologies like synthetic DNA vaccination that circumvent some of the issues associated with

traditional vaccines will likely prove critical. In addition, compounding factors that may influence immune outcome such as the microbiome both in the gut and skin must also be studied in greater detail. Recent clinical studies have suggested that the presence of certain bacteria in the gut is associated with favorable outcomes in patients receiving immunogenic chemotherapy (Routy et al. 2018). Other studies have also shown that a dysbiosis or overrepresentation of certain bacteria strains was negatively associated with favorable outcome. A recent clinical study found that volunteers with low pre-existing antibody titers to the influenza virus or vaccine that took a 5 day broad spectrum antibiotics course had impaired responses to an influenza vaccine (Hagan et al. 2019), further implicating the importance of the biome on vaccine induced immunity in humans. Further work needs to be performed to more fully understand the influence that the microbiome exerts on the immune system and vice versa, and the significance of this relationship in designing future therapies.

History of DNA vaccines: New and Improved

Following the initial reports of DNA's ability to be used as an immunogen for generating an immune response over twenty five years ago, significant work has been focused to realize DNA's intrinsic potential as a safe and potent vaccine platform in a variety of contexts, including infectious diseases and cancer applications. While an enormous amount of exciting preclinical animal model data has been generated, until recently, despite the platform's safety, translation from small animal models to larger animals with robust immunity, as well as in the clinic was not achieved. However, recent advancements, including improved technologies for DNA delivery, improved concentrated formulations, improved stability of product, rapid production, improved construct sequence design including genetic optimizations focusing on RNA changes as well as codon optimization, non native leader sequences, and the inclusion of genetic adjuvants, have begun to establish this new synthetic DNA platform as a serious partner for rapid development for

multiple applications and in particular for rapid protection against emerging infectious disease threats.

In the early 1990s, four separate groups reported that plasmid gene delivery resulted in *in vivo* expression and immune responses against the antigen. In 1992, Tang and Johnston reported the delivery of human growth hormone (HGH) DNA to the skin of mice using a gene gun (Tang, DeVit, and Johnston 1992), believing that this could be a useful technique for gene therapy, however the gene therapy approach was not effective as the plasmid delivery resulted in antibodies against the HGH encoded protein. Separately, at the Cold Spring Harbor vaccine meeting in 1992, Margaret Liu along with her colleagues at Merck (Ulmer et al. 1993), as well as Harriet Robinson (Fynan et al. 1993), from the University of Massachusetts, described DNA plasmid's ability to drive immune responses against influenza virus using plasmid delivered antigens, while David B Weiner reported that plasmids encoding constructs for HIV or tumor antigens could induce neutralizing antibody responses as well as CTL's resulting in protection against tumor challenge (B. Wang et al. 1993). These three reports were soon published and stood as evidence to the vaccine field that a new technology consisting of deceptively simple DNA delivery could serve as a simple immunization platform in a number of models. These early experiments in mice were to face immune potency issues over the next few years in larger animal models.

The vaccine field however, was excited by these initial studies. DNA vaccines have multiple conceptual advantages over traditional killed, live attenuated, and viral vector based vaccines (Table 1). DNA is simple to work with, allowing for relatively easy manipulation for a variety of applications. DNA vaccines are nonlive and nonreplicating, eliminating the risk of attenuation/reversion and also allow for safe delivery in high-risk populations, including persons who may be immunocompromised. DNA vectors are themselves not immunogenic, allowing for repeated administration without immune interference or concerns regarding previous viral

exposure limitations. In addition, DNA in theory can be manufactured to be more stable than traditional viral and killed vaccines thus possibly improving reliance on a complete cold chain, which in turn makes it an ideal candidate for important products developed for resource strained settings.

DNA vaccines contain antigen sequences that encode for a particular part of a pathogen or tumor, and are inserted into a mammalian plasmid expression vector. The vector now becomes the new vaccine. Following production, this plasmid vector can be delivered intradermally or intramuscularly, i.e. locally to tissues, where upon cell entry, some of the delivered plasmid will enter the nucleus of transfected cells and plasmid-encoded sequences will drive host cell transcription, producing the protein *in vivo*. This now *in vivo* produced foreign protein, can be expressed both in the transfected cells as well as released from these transfected cells to be recognized by immune cells. The protein can become subject to immune surveillance allowing for presentation of this now foreign antigen on the Class I and Class II antigen presenting systems. The native host system responds to this foreign antigen by eliciting a response including both antibodies (B cell responses) as well as cellular immunity (T cell responses), which can be protective in animal challenge models (Kutzler and Weiner 2008).

Due to the conceptual advantages in simplicity, production, and storage of DNA vaccines over traditional live as well as nonlive platforms, and the success seen in most small animal preclinical models, excitement regarding the outcome of the DNA platform in humans seemed all but assured. However, as early human clinical trials failed to display the same level of immune response observed in preclinical studies, concerns mounted. The platform was well tolerated in people, but these initial vaccines were poorly immunogenic in the clinic. These results soon repositioned DNA to take a backseat as a primary immune approach, and opened up a new secondary role for DNA vaccines as a component in prime boost model systems (Kardani, Bolhassani, and Shahbazi 2016; Woodland 2004; McCormack et al. 2008). In these systems,

DNA is used as an initial priming immunization to focus and jumpstart the immune response, and then either protein or viral vector is used in subsequent boosting immunizations. This combined approach led to greater immune responses compared to either platform alone and helped the viral vector approach partially avoid the host immune response.

Almost 20 years after their debut, the technology that underpins the first generations of DNA vaccines has been reexamined and reengineered with the goal of improved potency and consistent immune induction in the clinic. The initial vaccines utilized dilute formulations of DNA, limiting the DNA dose that could be delivered, thereby limiting the efficacy of the vaccine. Today, due to new formulations (Ferraro et al. 2011; Suschak, Williams, and Schmaljohn 2017; Grunwald and Ulbert 2015), much more highly concentrated SDNA plasmids are utilized, at doses upward of 10 mg/ml, which can increase vaccine efficiency. In addition to being more concentrated, newer formulations can be developed that are much more stable, reducing the need for complete cold chain transport, broadening the use of this approach in resource strained settings where total refrigeration or freezing may present challenges.

Genetic sequence optimization: moving on up to better expression

In addition to improving SDNA delivery concentrations, a vast amount of work has gone into optimizing the genetic sequence of these vaccines. One approach has been to carefully select the antigen sequence to encode for codons that target preferred transfer DNAs (tDNAs) for any given species (Ravi Vijaya Satya, Amar Mukherjee, and Udaykumar Ranga 2003). It's thought that codons that target more abundant tDNAs are translated more efficiently and thus express more antigen, potentially increasing immunogenicity of the SDNA vaccine. RNA optimization has also enhanced SDNA vaccine improvement. By eliminating elements that encourage RNA secondary structures such as stem loops, which can stall and disrupt mRNA transport, the mRNA transcripts are stabilized and allow for high antigen expression.

Further plasmid modifications that have contributed to improved SDNA expression include the inclusion of a more efficient PolyA signal (Montgomery et al. 1993; Hartikka et al. 1996), which terminates transcription and allows mRNA to be exported out of the nucleus. The use of nonnative leader sequences, such as the efficient IgE leader sequence (Kutzler et al. 2005; S. Wang et al. 2006), can stabilize mRNA and increase translation efficiency, further enhancing antigen expression. The development of smaller plasmids has effectively increased plasmid concentration in a given volume, allowing for more SDNA to be delivered per "dose."

Electroporation Technology: an electric solution to an old delivery problem

The use of new, more potent delivery technologies combined with the new DNA formulations further advanced the field. Specifically, the use of newer and reengineered electroporation (EP) devices to enhance *in vivo* transfection of delivered DNA during immunization, can result in a 100-1000x increase in transfection efficiency (Sardesai and Weiner 2011). The application of an electric field immediately upon DNA injection enhances DNA uptake in two ways: EP creates transient pores in the membrane where the DNA can enter the cell, and also generates an electric field to drive the DNA into those cells as well. These activities combine to boost DNA uptake, creating a large bolus of foreign protein *in vivo,* ultimately driving improved immune responses against the vaccine. Although older electroporation technology was initially considered too harsh to routinely use in humans, advances in the EP field including computer driven devices, lowered voltages, resistance sensors with controlled current and timing settings, have all led to a more tolerable experience in people, and dramatically improved vaccine take, making EP a viable candidate in vaccine development. As a consequence of these advances, delivery of DNA vaccines by EP in large animal models has led to increased cellular and humoral immune responses, rivaling those seen with viral vectors. Importantly, advanced EP that takes advantage of higher concentrated formulation and targets skin delivery may be particularly

relevant for emerging infectious diseases (EID) settings, where robust and rapid immune responses are necessary.

Harnessing the immune system's messengers as potential adjuvants to DNA vaccines

Adjuvants have had a long history in the vaccine field, and have been primarily used to increase immunogenicity of various vaccines. Formulated adjuvants can function through a number of mechanisms, including enhanced antigen uptake and presentation, antigen depot formation, and activation of the innate immune system. Alum is currently the most widely used adjuvant in licensed vaccines, and while it has been successful at increasing vaccine responses, alum mostly enhances Th2 humoral responses, thus limiting its use in vaccine platforms where enhanced cellular responses are desired. Other materials, including oil-in-water emulsions have also been studied as potential adjuvants. AS03, produced by GSK (Garçon, Vaughn, and Didierlaurent 2012), contains α-tocopherol and squalene, and has been shown to enhance vaccine specific humoral immune responses by increasing antigen uptake and presentation. In the clinic, AS03 was incorporated in the pandemic H1N1/2009 vaccine and showed increased vaccine immunogenicity compared to non-adjuvanted vaccine. AS04, an adjuvant that is comprised of monophosphoryl lipid A and alum, is licensed and used in the human papillomavirus (HPV) vaccine Cervarix. A number of nontraditional adjuvants are being investigated as well, including pathogen-recognition receptor (PRR) agonists, nanoparticles, liposomes, and gene-encoded adjuvants (Lee and Nguyen 2015). PRR agonist adjuvants, including Toll-like receptor (TLR) ligands, exploit innate immune signaling, jumpstarting the body's first line of defense. This in turn can work in concert with the adaptive immune system to generate lasting memory against the antigen. TLRs are generally expressed by macrophages and dendritic cells that are constantly surveying for conserved pathogen associated molecular patterns (PAMPs) derived from microbes that breach initial physical barriers (Coffman, Sher, and Seder 2010). Their role for enhancement of gene encoded vaccines remains to be determined.

Gene encoded adjuvants, such as cytokine DNA sequences, have also been studied as potential adjuvants for DNA vaccines. Cytokines are small proteins expressed by leukocytes and other non-immune cells that modulate the immune system. By delivering cytokines at the site of vaccination, it is possible to specifically tailor the immune response to adequately respond to future challenges. Gene encoded cytokine delivery allows the cytokine to be present at the same time as the antigen, increasing the likelihood that the cytokine can act within the window period where initial immune responses are occurring. Another advantage of delivering cytokines at the site of immunization is the avoidance of systemic exposure, which can reduce the risk of systemic side effects. A vast number of cytokines have been studied as potential adjuvants, including IFN-α, GM-CSF, Flt-3 ligand, IL-18, IL-21, IL-15, IFN-γ, IL-12, and IL-2 (Taylor 1995; Tovey and Lallemand 2010), in a number of experimental models; importantly, much work still needs to be done in the vaccine field regarding these cytokines as potential adjuvants.

In the context of DNA gene encoded adjuvants, Interleukin 12 (IL-12) has established an important potency track record for several years and is the most studied cytokine DNA adjuvant in the clinic. IL-12 is a pro-inflammatory cytokine primarily secreted by dendritic cells that links the innate and adaptive immune response, promoting enhanced Th1 cellular responses. Given its potent Th1 activation, there has been a lot of interest in using IL-12 as an adjuvant in various vaccine platforms, most notably in cancer trials. Early trials where IL-12 protein was delivered systemically resulted in major side effects, limiting potential use. However, local delivery of plasmid encoded IL-12 (pIL-12) does not drive systemic toxicity in the clinic (Schadeck et al. 2006; Sin et al. 1999; Tugues et al. 2015; Cha and Daud 2012; Kalams et al. 2012). Multiple trials have studied pIL-12 as an adjuvant administered as DNA formulated as part of the plasmid vaccine. In this delivery, the IL-12 adjuvanted vaccines have been well tolerated and some of these studies have seen clear immune improvements from the presence of IL-12 adjuvant. A recent study by Kalams et al is illustrative (Kalams et al. 2013). In this study the combination of EP + IL-12 drove much improved T cell responses for both CD4 and CD8 immunity. This HIV

Vaccines Trials Network (HVTN) study that combined plasmid encoded human immunodeficiency virus (HIV) antigens encoding Gag/Pol and Env +plasmid IL-12 +EP described that the combination approach resulted in overall T cell response rates of 90%, which were similar to combination vaccine studies that required boosting with viral vectors. As another example, a clinical trial that used a multi antigen HIV DNA prime and vesicular stomatitis virus (VSV) Gag protein boost with increasing doses of plasmid DNA IL-12 found that there were increased $CD8^+$ T cell responses in people adjuvanted with plasmid IL-12 compared to those whose vaccine was not adjuvanted (S. S. Li et al. 2017). The $CD8^+$ T cell responses observed post boost were also enhanced compared to non-adjuvanted groups. As more clinical trials are performed testing IL-12's potential as an adjuvant in the DNA + EP setting in additional disease models, we will gain additional insight into the immune activity of these combined approaches.

This initial data has encouraged the study of many additional cytokines, including those whose functions are less well understood, but appear to be interesting as potential adjuvants for DNA vaccines. Villarreal et al. showed that IL-33, an alarmin that is thought to alert the immune system to different stimuli and tissue damage was able to act as an immune adjuvant and enhance immune responses in tuberculosis, LCMV, and cancer animal models (Villarreal et al. 2014; Villarreal, Siefert, and Weiner 2015; Villarreal and Weiner 2014). Villarreal further advanced the field in showing that although IL-33 was traditionally thought to only drive Th2 humoral responses, it has the ability to drive Th1 and $CD8^+$ cellular functions as well.

There is a lot of exciting research currently being done in the field to find new potent adjuvants to boost immune responses to vaccines, including research on adjuvant delivery systems, combination studies and plasmid codelivery (Saade and Petrovsky 2012; Temizoz, Kuroda, and Ishii 2016). Adjuvants have the potential to reduce vaccine dose and frequency, overcome immune senescence, and allow for new vaccine targets. A special focus on adjuvants that can be delivered to the skin may prove advantageous, given the number of antigen presenting

Langerhan cells found in this tissue as well as the critical immune interactions constantly occurring at this site.

The microbiome and vaccine induced immunity

Appreciation has grown over the last 20 years that the human body provides a home to more than ~60 trillion microorganisms, of which at least half are bacterial. This is collectively referred to as the microbiome. It has become apparent that the microbiome is a major important piece of our biology that impacts health on many levels, and this eclectic collection of microorganisms is critical for maintaining homeostasis in the human system. As more is understood about the microbiome, thoughts about plasmid delivery for prophylactic and therapeutic applications are also evolving. On average, the bacteria that comprise our microbiomes will have a life span of between 12 and 24 hours, thus continually exposing the host to plasmid and bacterial DNA naturally. The FDA has expressed some concern regarding the delivery of DNA as a vaccine platform, however the small amount of DNA that we additionally deliver in a DNA vaccine is likely of little consequence in this grand scheme.

One particular area of interest for the vaccine field is the role that the microbiome may play in vaccine-induced immune responses (Deriu et al. 2016; Shi et al. 2017; Ichinohe et al. 2011; Round and Mazmanian 2009). Recent data suggests that the types of bacteria and relative amounts of each type of bacteria may directly impact the efficacy of vaccines. Microbial cells are primarily found in the intestinal tract, as well as the skin, bronchial and genital tract. Studies using germ free mice or those treated with antibiotics to deplete intestinal bacteria have shown defective innate immune responses to infectious diseases including influenza. Upon microbiome restoration, proper immune responses were also restored. These studies also showed the importance of the strain of bacteria. Mice that were colonized with flagellated E. coli mounted the appropriate immune response against influenza A, whereas mice colonized with non-flagellated E. coli did not. Given that the microbiome is largely established within the first 6 months of life,

around the same time that many vaccines are first administered, additional study of these early colonizers in this context will be important. In a striking study, researchers compared the microbiome of infants from Ghana and the Netherlands that were vaccinated against rotavirus and found that the Dutch infants were generally able to mount a strong immune response to the vaccine, while many Ghanaian infants were unable to do so (Harris et al. 2017). Of the Ghanaian infants that did mount an immune response, their microbiomes were much more similar to the Dutch infants compared to the microbiomes of the nonresponders. The implications of this study suggest that the microbiome may play a significant role in vaccine outcomes in diverse populations. In a clinical study published this year examining the impact of the microbiome in influenza vaccine induced immune responses, Hagan et al found that adults with high pre-existing titers to the vaccine did not exhibit impaired antibody responses after taking a course of broad spectrum antibiotics, despite a 10,000 fold decrease in gut bacterial load (Hagan et al. 2019). However, in adults that had low titers to the vaccine beforehand, antibody responses to a specific strain in the vaccine were greatly impaired. Interestingly, the study also found that these individuals exhibited similar inflammatory responses as those observed in older people that struggle to adequately generate robust immunity to the influenza vaccine. As the number of clinical trials that test the impact of more tolerable and less invasive vaccine programs such as intradermal vaccine delivery increases, understanding the potential immune interactions between local bacteria on the skin and the immune cells critical in the primary immune response will become increasingly important.

As the era of therapeutic vaccine mediated approaches for cancer is well underway, the influence of the microbiome cannot be understated. Clinical studies that have evaluated the effectiveness of immune-checkpoint inhibitors that target PD-1 or CTLA-4 found a positive association between the presence of bacteria such as *Akkermansia muciniphila, Bifidobacterium* spp., and *Faecalibacterium* and anti-cancer outcomes (Routy et al. 2018). Characterization of the gut microbiome of patients with metastatic melanoma treated with anti-PD1 antibodies showed that

those who responded to the therapy had a greater abundance of bacteria from the Ruminococcaceae family. Future trials must be performed to determine whether the microbiome can influence therapeutic outcome in other disease models. In a clinical HPV DNA vaccine study, 50% of vaccinated women regressed their disease overall. 40% of these women actually eliminated the precancer, cleared their underlying HPV16/18 infection, and exhibited complete histopathologic regression compared to only 14.3% in the placebo group (Trimble et al. 2015). While this represents an important breakthrough as the first therapeutic vaccine to show efficacy against cervical intraepithelial neoplasia grade 2/3 (CIN2/3) associated with HPV16/18, there is still a lot of work needed to elucidate some of the differences between the women who responded and those that did not. Interestingly, some patients were able to regress, but did not clear the underlying infection. As the urogenital tract itself is home to a unique microbiome, a study of the bacteria populations in the patients who cleared and regressed, regressed but did not clear, or didn't respond is certainly worth investigating.

Lessons learned in rapid vaccine development in the midst of infectious outbreaks

Recent global events have highlighted the need for rapid, effective vaccine development for emerging infectious diseases. The World Health Organization (WHO) warned a decade ago that infectious pathogens were emerging and reemerging at rates unseen before. Traditional vaccines have been developed on the scale of years, which is not ideal in the midst of a sudden epidemic, as illustrated by the 2014-2016 Ebola outbreak. In response to this particular outbreak, many groups set out to create therapies and vaccines that could impact these outbreaks, treat those who were infected, or prevent transmission to those that were uninfected. The recent Zika emergency is a case in point. Very rapidly after the Zika epidemic was reported, a synthetic DNA vaccine was engineered to generate immunity against the Envelope protein of Zika and designed for skin delivery using high concentration formulations of the DNA in a very small volume (Muthumani et al. 2016). As part of the design, the vaccine contained sequences encoding the

precursor membrane (prM) region to help with transport and processing of the Envelope (E) antigen. After very encouraging preclinical data, the vaccine was moved to the clinic in just over 6 months and became the first Zika vaccine in human clinical testing (Figure 1). The clinical trial sought to answer whether there was some advantage to delivering the vaccine intradermally vs intramuscularly, and found that volunteers that were immunized in the skin had stronger antibody and cellular responses to the vaccine compared to those immunized in the muscle. The synthetic prME vaccine induced rapid seroconversion in greater than 95% of volunteers by two immunizations and 100% seroconversion after 3 immunizations (Tebas et al. 2017). Importantly, the induced antibodies from this study were able to protect immune deficient mice from a lethal Zika virus challenge by passive transfer, suggesting that the antibodies developed through vaccination in vaccine volunteers may be sufficient to protect against subsequent exposure to the virus. Additionally, T cell responses were induced in most vaccine recipients in this study, suggesting the activation of both arms of the adaptive immune system. The use of newer SDNA technologies for outbreak strategies that can be rapidly moved to the clinic appear to be finally establishing an important track record for safety, speed and immune potency. Furthermore, the relative ease of intradermal vaccine delivery will likely enhance vaccine uptake in a number of diverse settings.

Looking to the future

With increased globalization and climate change, novel infectious diseases are an expanding threat to previously unaffected areas, underscoring the need for rapid development of new vaccines. The National Institute of Allergy and Infectious Diseases (NIAID) Biodefense program maintains a record of infectious pathogens and diseases it considers top priorities, which paints a sobering picture of the work ahead for the field (Figure 2). In addition to newly emerging infections, some previously known pathogens can mutate to give rise to new strains that may

trigger pandemics. In tackling these pathogens, lessons learned from the Ebola, Zika, and MERS outbreaks can help guide future vaccine programs.

The SDNA platform has significant potential to contribute to rapidly impacting new outbreaks. Collective advancements to the platform, including higher concentrations of product and improved delivery methods for enhanced EP targeting ID space for example, have begun to alter DNA's reputation, supporting it as a viable candidate for prophylaxis and therapy options. The inherent properties of plasmid DNA production, including low manufacturing costs, excellent safety profile, rapid scale up potential, high immune response rate of vaccines and short time to clinic, are highly encouraging, especially as the number of efficacy trials is growing. As the platform continues to evolve and target discovery becomes more precise, the promise of this new generation of DNA technologies will be further tested and refined.

* Modified from: Louis L, Weiner DB. (2019). Rapid Synthetic DNA vaccine/immunotherapeutics for infectious disease and cancer targets. *Microbiome and Cancer*. Current Cancer Research (347-362)

Parameter	Live Attenuated Vaccine	Peptide/ Protein subunit	Bacterial Vector	Viral Vector	RNA Vector	DNA Vector	SynCon DNA
Synthetic	-	-	-	-	Yes	Yes/no	Yes
Rapid Simple clinical production	-	-	-	-	-	Yes	Yes
Safety	Yes - some consideration	Yes -	With consideration	Yes- some consideration	Yes – IFN responses	Yes	Yes
Antibodies	Yes	Peptide-no Protein- yes	No to low	Yes	No to low	No to low	Yes
CTL-Killing - humans	Yes	Primarily low to no	low	Yes- particularly Ad vectors	Yes	Low	Yes- potent
In vivo Antigen expression	Yes	-	Yes	Yes	Yes	Yes	yes
Off target immune antigens	Yes	-	Yes	Yes	No/yes for replicating systems	-	-
Repeat Boosting	Poor to no	Yes	Poor	Poor	Yes	Yes	Yes
Simple Antigen combinations	-	-	-	-	Yes	Yes	Yes
Immune Potency	Yes	Limited	Limited	Yes	Early data	-	Yes

Table 1. Comparison of Various Vaccine Platforms.

Table compares a number of parameters of different vaccine platforms.

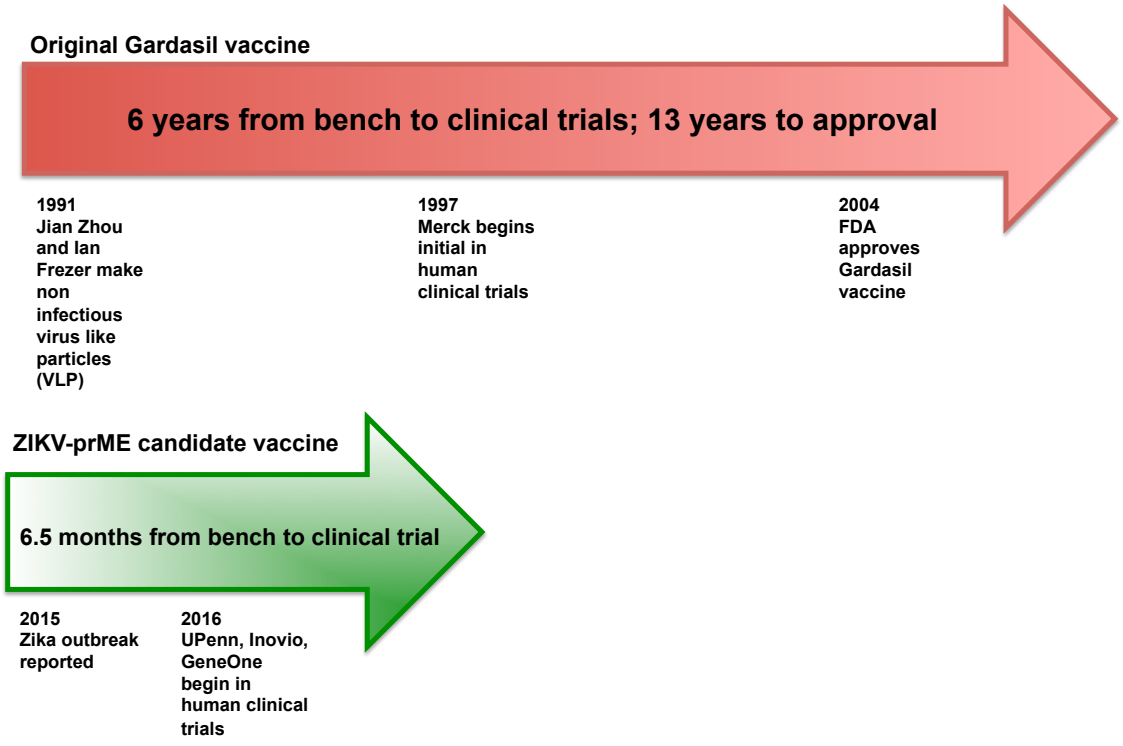


Figure 1. Timeline comparing the development of Gardasil and ZIKV prME vaccines.

This is an illustration of the major time points in the development of the original Gardasil vaccine. From the development of VLPs, it took 6 years to begin clinical trials, while it took six and a half months from the time of initial reports on Zika outbreak to a clinical trial testing the Zika SDNA vaccine, highlighting the potential of the DNA vaccine platform to rapidly develop candidate vaccines during outbreaks.

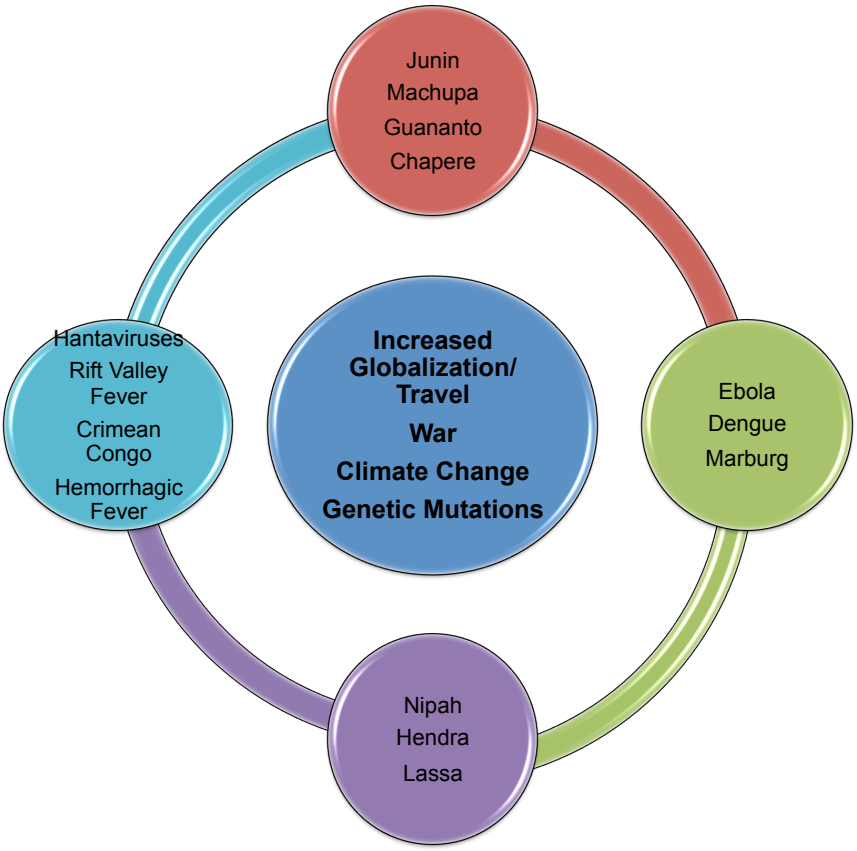


Figure 2. Diagram of emerging infectious diseases according to NIAID.

The diagram highlights a number of infectious pathogens and diseases that the National Institute of Allergy and Infectious Diseases (NIAID) Biodefense program considers top priorities. In addition to newly emerging infections, there are also a number of pathogens that have mutated to give rise to new strains that may trigger pandemics. Patterns of climate change, increased travel, as well as genetic mutations have all contributed to this growing list.

Goals of this thesis project

As the newer DNA technology continues to improve, there is a need to fully maximize its potential in previously under explored areas. DNA vaccination and immune response in the context of skin, particularly cellular responses, is one such example. One goal of my thesis project was to evaluate the potential of a skin derived cytokine, IL-36, to act as an adjuvant to complement and enhance DNA vaccine-induced cellular responses. The need for novel adjuvants that can boost $CD8^+$ T cell responses will continue to grow, and the concept of skin specific adjuvants for DNA has not been previously explored. These adjuvants may be especially useful in cancer applications where $CD8^+$ T cells are critical for tumor control, or for vector borne infections, which frequently originate at cutaneous sites. For initial studies, we encoded both full length and truncated forms of IL-36 in a plasmid and assessed whether there was a difference in the immune responses observed following vaccination given reports of increased bioactivity upon truncation. We then tested the cytokine with a number of disease antigens to assess whether it could be used in a variety of vaccine models to boost immunity, and lastly observed whether any enhanced immunity provided by IL-36 could protect in an established Zika challenge model. We further sought to examine whether cellular responses induced by IL-36 could provide protection in a model where antibody mediated protection is thought to be most important.

The immune responses generated by intradermal DNA vaccination, especially cellular responses, have not been fully explored. The Zika clinical study mentioned earlier, as well as an RSV DNA vaccine study in the cotton rat, both used an intradermal delivery approach, however these studies primarily focused on induced humoral responses. Another major goal of my thesis project was to examine the cellular responses generated during intradermal DNA vaccination and to investigate whether these immune responses generated would be protective in a challenge model where cellular responses are critical. For our initial studies, we assessed the immune responses generated by intradermal vaccination of an established HIV DNA vaccine to ensure the broad application of this strategy across multiple antigens and disease models. I also focused on the

development of a new consensus DNA vaccine against a newly described Leishmania antigen, which has been reported to have ability to induce protective immune responses against challenge as a peptide-based immunogen. This early study was exciting but not confirmed in a follow up study. Accordingly, I was interested in assessing whether intradermal delivery of a DNA encoded vaccine could induce potent immune responses at the site of immunization and impact protection in an important pathogen challenge.

Ultimately, the main goal of my thesis project was to determine if the skin could be mined, both as a site of vaccination and as a provider of immune messengers, to further advance and improve the DNA vaccine platform as the demand for new technologies to address ever more challenging targets increases.

CHAPTER 2- MATERIALS AND METHODS

***Cell lines*.** HEK293T and U2OS cells were purchased from ATCC and grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% heat inactivated FBS and antibiotics (Invitrogen; 100 units/mL Penicillin and 100 μg/mL streptomycin).

***Plasmid constructs*.** The HIV consensus clade C Envelope (Env) vaccine used in the intradermal and intramuscular studies was generated based on the sequences retrieved from HIV databases (http://www.hiv.lanl.gov). To produce a CCR5-tropic version of the HIV-1 envelope, six important amino acids in V3 loop were designed according to the sequences of early transmitter isolates. Six amino acids in V1 loop and three amino acids in V2 loop were deleted. The cytoplasmic tail region was removed to promote higher expression of Env protein. The gp120/41 Env cleavage site was incorporated to promote proper folding of the synthetic Env protein. A more efficient IgE leader sequence was added to the N-terminus of the gene. The transgene was codon and RNA optimized for expression in human and mouse, synthesized by Genscript and cloned into modified pVax1 mammalian expression vectors (Invitrogen) under the control of the human cytomegalovirus (CMV) immediate-early promoter.

The DNA plasmids IDM2 and PB encode Leishmania Phosphoenolpyruvate carboxykinase (PEPCK) and represent consensus sequences of PEPCK from a number of Leishmania parasite strains. IDM2 is a consensus of PEPCK sequences from Leishmania parasites **I**nfantum, **D**onovani, **M**ajor, and **M**exicana [Uniprot accession numbers A4I2Y7, E9BJI0, E9ADF9, E9AZ81]. PB is a consensus of PEPCK sequences from Leishmania species **P**anamensis and **B**raziliensis [Uniprot accession numbers A0A088RTT4, A4HFV1]. A more efficient IgE leader sequence was added to the N-terminal region of both genes. The transgenes were codon and RNA optimized for expression in human and mouse, synthesized by Genscript and cloned into modified pVax1

mammalian expression vectors (Invitrogen) under the control of the human cytomegalovirus (CMV) immediate-early promoter.

The DNA plasmid used in the influenza study targets the hemagglutinin (HA) protein of H1N1 influenza A/PR/8/34 (PR8). The HA sequence was codon and RNA optimized for expression in humans using GeneOptimizer sequence analysis software (Life Technologies), synthesized and cloned by Aldevron into the pVax mammalian expression vector (Invitrogen) under the control of the cytomegalovirus immediate-early promoter (CMV).

The DNA plasmid used in the Zika studies (ZIKV-prME) encodes full-length precursor membrane (prM) plus Envelope/Env (E). A consensus strategy was used and the consensus sequences were determined by the alignment of current Zika virus (ZIKV) prME protein sequences. A more efficient IgE leader sequence was added to the N-terminal region of the gene. ZIKV-prME was codon and RNA optimized for expression in humans, synthesized and cloned by Genscript into pVax1 mammalian expression vector (Invitrogen) under the control of the cytomegalovirus immediate-early promoter (CMV).

For the IL-36 cytokine studies, the full-length DNA plasmids encode murine IL-36 alpha, beta, and gamma [Uniprot accession numbers Q9JLA2-1, Q9D6Z6-1, Q8R460-1]. A more efficient IgE leader sequence was added to the N-terminal region of all three genes. The transgenes were codon and RNA optimized for expression in human and mouse, synthesized by Genscript and cloned into modified pVax1 mammalian expression vectors (Invitrogen) under the control of the human cytomegalovirus immediate-early promoter (CMV). These full-length optimized IL-36 cytokine plasmids are known henceforth as opt-36α, opt-36β, and opt-36γ. Work by Towne et al. has suggested the need for truncation of IL-36 cytokines nine amino acids N-terminal to a conserved *A-X-Asp* motif, for full bioactivity. The second set of IL-36 plasmids was truncated according to this data and they are henceforth known as opt-36αt, opt-36βt, and opt-36γt. These inserts were modified as previously mentioned for full-length IL-36 plasmids.

***Leishmania parasites*.** *Leishmania major* parasites (WHO/MHOM/IL/80/Friedlin) parasites were grown in Schneider's insect medium (Invitrogen) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Metacyclic enriched promastigotes were used for infection.

Zika virus. Zika virus strain PR209 (Bioqual, MD) was amplified in Vero cells and stocks were titred by standard plaque assay on Vero cells.

Western Blots. For transfection of PEPCK plasmids, HEK293T cells were grown to 80% confluence in 6 well flat bottom tissue culture plates and transfected with 2 μg of HA tagged IDM2 or PB. The cells were collected 2 days after transfection, washed twice with PBS and lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA). Gradient (4–12%) Bis-Tris NuPAGE gels (Life Technologies, Carlsbad, CA) were loaded with transfected cell lysates and transferred to PDVF membrane. The membranes were blocked in PBS Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature. To detect plasmid expression, anti-HA (A01244 Clone 5E11D8, GenScript) antibody was diluted 1:1000 and anti–β-actin antibody diluted 1:5000 in Odyssey blocking buffer with 0.2% Tween 20 (Bio-Rad, Hercules, CA) and incubated with the membranes overnight at 4 °C. The membranes were washed with PBST and then incubated with the appropriate secondary antibody (goat anti-mouse IRDye680CW; LI-COR Biosciences) at 1:15,000 dilution in Odyssey Blocking Buffer for 1 hour at room temperature. After washing, the membranes were imaged on the Odyssey infrared imager (LI-COR Biosciences).

For transfection of opt-36αt, opt-36βt, and opt-36γt plasmids, U2OS cells were grown to 80% confluence in 6 well flat bottom tissue culture plates and transfected with 2 μg of opt-36αt, opt-36βt, and opt-36γt. The cells were collected 2 days after transfection, washed twice with PBS and lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA). Gradient (4–12%) Bis-Tris NuPAGE gels (Life Technologies, Carlsbad, CA) were loaded with transfected cell lysates and

transferred to PDVF membrane. The membranes were blocked in PBS Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature. To detect plasmid expression, anti-HA (A01244 Clone 5E11D8, GenScript) antibody was diluted 1:1000 and anti–β-actin antibody diluted 1:5000 in Odyssey blocking buffer with 0.2% Tween 20 (Bio-Rad, Hercules, CA) and incubated with the membranes overnight at 4 °C. The membranes were washed with PBST and then incubated with the appropriate secondary antibody (goat anti-mouse IRDye680CW; LI-COR Biosciences) at 1:15,000 dilution in Odyssey Blocking Buffer for 1 hour at room temperature. After washing, the membranes were imaged on the Odyssey infrared imager (LI-COR Biosciences).

Immunofluorescence Assay (IFA). For the immunofluorescence assay, HEK293T cells were grown in 6 well tissue culture slides and transfected with 2 μg of IDM2 or PB. Two days after transfection, the cells were fixed with 4% paraformaldehyde for 15 min. Nonspecific binding was then blocked with normal goat serum diluted in PBS at room temperature for 1 h. The slides were then washed in PBS for 5 min and subsequently incubated with sera from IDM2 and PB immunized mice at a 1:100 dilution overnight at 4 °C. The slides were washed as described above and incubated with appropriate secondary antibody (goat anti-mouse IgG-AF488, Sigma, St Louis, MO) at 1:200 dilutions at room temperature for 1 h. After washing, DAPI (Millipore Sigma, Burlington, MA) was added to stain the nuclei of all cells following manufacturer's protocol. Wells were washed and maintained in PBS, and observed under a microscope (EVOS Cell Imaging Systems; Life Technologies).

For IFA analysis of opt-36αt, opt-36βt, and opt-36γt, U2OS cells were grown in 6 well tissue culture slides and transfected with 2 μg of HA-tagged opt-36αt, opt-36βt, and opt-36γt. Two days after transfection, the cells were fixed with 4% paraformaldehyde for 15 min. Nonspecific binding was then blocked with normal goat serum diluted in PBS at room temperature for 1 h. The slides were then washed in PBS for 5 min and subsequently incubated with anti-HA antibody at a

1:1000 dilution overnight at 4 °C. The slides were washed as described above and incubated with appropriate secondary antibody (goat anti-mouse IgG-AF488, Sigma, St Louis, MO) at 1:200 dilutions at room temperature for 1 h. After washing, DAPI (Millipore Sigma, Burlington, MA) was added to stain the nuclei of all cells following manufacturer's protocol. Wells were washed and maintained in PBS, and observed under a microscope (EVOS Cell Imaging Systems; Life Technologies).

HIV gp120 protein binding ELISA. The ELISA was performed using 1 μg/ml HIV consensus C gp120 (Immune Technology Corp., New York, NY) in PBS with 0.5% Tween 20 (PBS-T). After a blocking step, serum from immunized mice was diluted to 1:50 and then 4-fold from there in 1% FBS in PBS-T. Each sample was run in duplicate. After a 1-h incubation, plates washed and incubated with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) at a 1:5,000 dilution in 1% FBS in PBS-T. Plates were then developed with SigmaFast OPD for 8-10 minutes and the OD_{450} values were obtained.

Influenza hemagglutinin protein binding ELISA. Plates were coated with 1 μg/ml of hemagglutinin ((H1N1) (A/New Caledonia/20/99) Immune Technology Corp.) in PBS. After a blocking step, serum from immunized mice was diluted to 1:50 and then 4-fold from there in 1% FBS in PBS-T. After a 1-h incubation, plates were washed five times with PBS-T washed and incubated with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) at a 1:5,000 dilution in 1% FBS in PBS-T. Plates were then developed with SigmaFast OPD for 8-10 minutes, and the OD_{450} values were obtained.

Influenza hemagglutinin avidity index ELISA. Plates were coated with 1 μg/ml of hemagglutinin ((H1N1) (A/New Caledonia/20/99) Immune Technology Corp.) in PBS. After a blocking step, serum from immunized mice was diluted to 1:50 and then 4-fold from there in 1% FBS in PBS-T. After a 1-h incubation, plates were washed five times with PBS-T. Half of the wells for each sample were incubated with denaturing reagent (8 M urea) for 5 min while the others

were incubated with PBS. Plates were washed and incubated with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) at a 1:5,000 dilution in 1% FBS in PBS-T. Plates were then developed with SigmaFast OPD for 8-10 minutes, and the OD_{450} values were obtained. The avidity index was determined by dividing the OD_{450} values of the treated samples by those of the untreated samples and multiplying by 100.

HIV ELISpot Assay. Precoated anti-IFN-γ ninety-six well plates (MabTech, Cincinnati, OH) were used to quantify IFN-γ responses to vaccine. Single-cell suspensions of splenocytes were made by homogenizing and processing the spleens through a 40 μm cell strainer. Cells were then re-suspended in ACK Lysing buffer (Gibco™) for 5 min to lyse red blood cells before two washes with PBS and final re-suspension in RPMI complete media (RPMI 1640+10% FBS+1% penicillin–streptomycin). Two hundred thousand splenocytes were added to each well and stimulated overnight at 37°C in 5% CO_2 with R10 (negative control), concanavalin A (3 μg/ml; positive control), or specific HIV Env Clade C peptides (NIH AIDS Reagent Program). Peptide pools consisted of 15-mer residues overlapping by 11 amino acids, representing the entire protein consensus sequence of HIV-1 clade C were obtained from NIH AIDS Research and Reference Reagent Program. The Env peptides were pooled at a concentration of 2 ug/ml/peptide into 4 pools as antigens for specific stimulation of IFN-γ release. After 18 hours of stimulation, the plates were washed and developed following manufacturer's protocol. The plates were then rinsed with distilled water and dried at room temperature overnight. Spots were counted by an automated ELISpot reader (Cellular Technology Ltd.).

Influenza ELISpot Assay. Precoated anti-IFN-γ ninety-six well plates (MabTech, Cincinnati, OH) were used to quantify IFN-γ responses to vaccine. Single-cell suspensions of splenocytes were made by homogenizing and processing the spleens through a 40 μm cell strainer. Cells were

then re-suspended in ACK Lysing buffer (Gibco^{TM}) for 5 min to lyse red blood cells before two washes with PBS and final re-suspension in RPMI complete media (RPMI 1640+10% FBS+1% penicillin–streptomycin). Two hundred thousand splenocytes were added to each well and stimulated overnight at 37°C in 5% CO_2 with R10 (negative control), concanavalin A (3 μg/ml; positive control), or 15 mer influenza hemagglutinin peptides overlapping by 11 amino acids spanning the length of the consensus H1 hemagglutinin protein (GenScript). After 18 hours of stimulation, the plates were washed and developed following manufacturer's protocol. The plates were then rinsed with distilled water and dried at room temperature overnight. Spots were counted by an automated ELISpot reader (Cellular Technology Ltd.).

Leishmania ELISpot. Precoated anti-IFN-γ ninety-six well plates (MabTech, Cincinnati, OH) were used to quantify IFN-γ responses to vaccine. Single-cell suspensions of splenocytes were made by homogenizing and processing the spleens through a 40 μm cell strainer. Cells were then re-suspended in ACK Lysing buffer (Gibco^{TM}) for 5 min to lyse red blood cells before two washes with PBS and final re-suspension in RPMI complete media (RPMI 1640+10% FBS+1% penicillin–streptomycin). Two hundred thousand splenocytes were added to each well and stimulated overnight at 37°C in 5% CO_2 with R10 (negative control), concanavalin A (3 μg/ml; positive control), or 15 mer IDM2 or PB peptides overlapping by 9 amino acids spanning the entire length of the consensus protein (Genscript). After 18 hours of stimulation, the plates were washed and developed following manufacturer's protocol. The plates were then rinsed with distilled water and dried at room temperature overnight. Spots were counted by an automated ELISpot reader (Cellular Technology Ltd.).

Zika ELISpot Assay. Precoated anti-IFN-γ ninety-six well plates (MabTech, Cincinnati, OH) were used to quantify IFN-γ responses to vaccine. Single-cell suspensions of splenocytes were made by homogenizing and processing the spleens through a 40 μm cell strainer. Cells were then re-suspended in ACK Lysing buffer (Gibco^{TM}) for 5 min to lyse red blood cells before two

washes with PBS and final re-suspension in RPMI complete media (RPMI 1640+10% FBS+1% penicillin–streptomycin). Two hundred thousand splenocytes were added to each well and stimulated overnight at 37°C in 5% CO_2 with R10 (negative control), concanavalin A (3 μg/ml; positive control), or 15 mer Zika peptides overlapping by 9 amino acids spanning the length of the ZIKV-prME protein. After 18 hours of stimulation, the plates were washed and developed following manufacturer's protocol. The plates were then rinsed with distilled water and dried at room temperature overnight. Spots were counted by an automated ELISpot reader (Cellular Technology Ltd.).

Flow cytometry. For intracellular cytokine staining, two million cells were stimulated in 96-well plates with overlapping peptide pools of HIV, influenza, Leishmania, or Zika protein as described in the ELISpot section, media alone (negative control) and phorbol 12-myristate 13-acetate (PMA) and ionomycin (BD Biosciences, San Jose, CA) (positive control) for 6 hours at 37 °C+5% CO_2 in the presence of GolgiPlug and GolgiStop™ (BD Biosciences). After 6 hours, cells were collected and stained in FACS buffer with a panel of surface antibodies containing live dead eFluor V450, FITC anti-CD4, Alexa Fluor 700 anti-CD44, and APC-Cy7 anti-CD8 for 30 min at 4 °C. Cells were washed and then fixed with Foxp3/Transcription Factor Fixation/Permeabilization (ThermoFischer Scientific) for 20 min at 4 °C. Cells were washed with Perm/Wash buffer before intracellular staining with PE-Cy7 anti-IL-2, PerCP-Cy5.5 anti-CD3ε, PE anti- TNFα, and APC anti-IFNγ for 1 hour at 4 °C. Cells were then washed with Perm/Wash buffer before suspension in Perm/Wash buffer and acquisition on a BD LSRII. All results were analyzed using FlowJo™ v.10.0 (TreeStar).

Animals. Wildtype C57BL/6 and Balb-C mice were purchased from Jackson and Charles River Laboratories. IFNAR $^{-/-}$ mice lacking the IFN-α/β receptor were bred and housed at the Wistar Institute Animal Facility. All animal housing and experimentation were in compliance with the

guidelines set by the NIH, the University of Pennsylvania School of Veterinary Medicine and the Wistar Institutional Animal Care and Use Committee (IACUC).

Animal Injection Studies. For intramuscular (IM) vaccination studies, mice were administered a 30 μl injection of plasmid into the tibialis anterior (TA) muscle followed by *in vivo* electroporation (EP) using a CELLECTRA adaptive constant current electroporation device (Inovio Pharmaceuticals Inc.). For intradermal (ID) vaccination studies, mice were administered a 30 μl injection of plasmid into the shaved abdominal flank skin to form a wheel (Mantoux injection) followed by *in vivo* surface electroporation (SEP) (Inovio Pharmaceuticals Inc.). The epidermal targeting surface EP (SEP) device consists of an electrode array made up from a 4x4 array of gold-plated trocar needles of 0.43 mm diameter at a 1.5 mm spacing, which was pressed down on the surface of the skin above the intradermal wheel in a manner in which all electrodes across the array made contact with the surface of the skin.

Flank skin isolation: Flank skin was shaved using an electric trimmer equipped with a two-hole precision blade (Wahl). A section of dermis was excised, and then minced with a sterile scalpel blade into ~2-mm sections. Flank sections were incubated in RPMI containing 250 μg/ml Liberase TL for 120 min, with vortexing every 30 min. The resulting solution was passed through a 40-μm cell strainer and resuspended in complete RPMI (cRPMI), which is supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 55 μM 2-mercaptoethanol.

Leishmania major Challenge Studies. L. major challenge studies were performed either one-week post final vaccination (acute time point) or six weeks post-final vaccination (memory). Mice were infected in the ear with 2 million *L. major* parasites. Immune mice group were infected with *L. major* parasites 12 weeks before subsequent challenge. For ear preparation dorsal and ventral layers of the infected ear were separated and incubated in RPMI (Invitrogen) with 250 μg/ml Liberase TL (Roche, Basel, Switzerland) for 90 min at 37°C in 5% CO_2. Ears were dissociated

using a 40-μm cell strainer (BD) and resuspended in RPMI media containing 10% FBS. Parasite burden from ear was calculated by serial 2-fold dilution in 96-well plates of G418 sulfate (CSM) and incubated at 26°C. The number of viable parasites was calculated from the highest dilution at which parasites were observed 7 days into culture.

Zika Challenge Studies. IFNAR^{-}/$^{-}$ mice were challenged with 1×10^{5} PFU ZIKV-PR209 virus two weeks post vaccination. Post challenge, the animals were weighed daily. In addition, they were observed for clinical signs of disease twice daily (decreased mobility; hunched posture; hind-limb knuckle walking (partial paralysis), paralysis of one hind limb or both hind limbs). The criteria for euthanasia on welfare grounds consisted of 20% weight loss or prolonged paralysis in one or both hind limbs.

Statistical Analysis. All graphs were prepared using GraphPad Prism 8 (GraphPad Software). Survival data were expressed using Kaplan-Meier survival curves. A modified one-way ANOVA test with Tukey posthoc test was used to determine differences between experimental groups.

"*Knowledge is power. Information is liberating. Education is the premise of progress, in every society, in every family*." -Kofi Annan

Scene: Lumena, age 23 preparing to start the Gardasil regimen so she can be one less woman who may have to deal with cervical cancer caused by HPV.

So what brings you in today for the Gardasil vaccine, Lumena? (nurse practitioner at Student Health)

I keep seeing those "one less, one less, I want to be one less woman who will get cervical cancer commercials" and I figure as much as I hate needles, I should probably get a vaccine that will help prevent cancer. (Lumena, trying to act like the adult that I am)

Great. Let's talk about what the vaccine is and some things you should be aware of. First, it is a 3-dose vaccine…. (nurse practitioner making sure I am informed)

Wow…. 3 shots huh? (Lumena, crestfallen at the thought of coming back two more times for this vaccine, wondering why so many shots can't be one and done)

CHAPTER 3- Designed DNA encoded IL-36 gamma acts as a potent molecular adjuvant enhancing Zika synthetic DNA vaccine induced immunity and protection in a lethal challenge model

Introduction

Identification of novel molecular adjuvants which can boost and enhance vaccine-mediated immunity and provide dose sparing potential against complex infectious diseases and for immunotherapy of cancer is likely to play a critical role in the next generation of vaccines. Given the number of challenging targets for which no or only partial vaccine options exist, adjuvants that can address some of these concerns are in high demand. Here, we report that a designed truncated IL-36 gamma encoded plasmid can act as a potent adjuvant for several DNA encoded vaccine targets including HIV, influenza, and Zika in immunization models. We further show that the truncated IL-36 gamma (opt-36γt) plasmid provides improved dose sparing as it boosts immunity to a suboptimal dose of a Zika DNA vaccine resulting in potent protection against a lethal Zika challenge.

The most successful approach to controlling infectious diseases on a global scale has been through vaccination. Vaccines have led to control, eradication or near eradication of several infectious diseases, positively impacting both human longevity and the quality of life. However, much work remains in this area. For many targets, current studies have suggested the need for adjuvants, which can provide a number of benefits including improved vaccine effectiveness, as discussed in several papers (Lahiri, Das, and Chakravortty 2008; Mosca et al. 2008; McKee, Munks, and Marrack 2007). Adjuvants can boost overall immune responses to a specific vaccine, thereby requiring either a lower dose or fewer immunizations, improving protection and compliance as well as increasing the global vaccine supply for a particular product (Shah, Hassett, and Brito 2017). Adjuvants can also help skew and tailor the immune response, which

may be useful in scenarios where specific correlates of protection are understood (Podda 2001; Khurana et al. 2010). Furthermore, adjuvants can boost immunity and shorten time to induce a protective vaccine response in populations that traditionally have a difficult time mounting protective responses, including the elderly and immunocompromised patients (Reed, Orr, and Fox 2013). Alum, the most widely used adjuvant among current licensed vaccines, is well documented to enhance humoral immunity (Wen and Shi 2016). Newer vaccine adjuvants including MF59 and the Adjuvant Systems group 03 and 04 (AS03, AS04) have also been licensed and shown to improve antibody responses to antigens as well as provide dose sparing among other benefits for humoral responses (Wilkins et al. 2017). Shingrix, the latest vaccine developed by GSK and approved to protect against reactivation of herpes zoster and postherpetic neuralgia (shingles), is a recombinant vaccine made of glycoprotein E and ASO1 adjuvant, a mixture of both MPL and QS-21, a saponin (Bharucha, Ming, and Breuer 2017). This vaccine demonstrated an efficacy of over 95% against herpes zoster, with greater efficacy compared to a live attenuated vaccine, ZostaVax, highlighting the impact that adjuvants can have on vaccine outcomes. However, in spite of this success, there is still a major need in the clinic for adjuvants that can improve CTL responses (Mosca et al. 2008). Some of the exciting work being done in this field includes nontraditional adjuvants such as pathogen-recognition receptor (PRR) agonists, liposomes, nanoparticles, and gene encoded cytokine adjuvants that can potentially jumpstart the innate immune system and work in concert with the adaptive immune arm to drive lasting memory against antigen.

The majority of the work done investigating PRR agonists has focused on Toll-like receptor ligands (TLRs). TLRs are generally expressed by macrophages and dendritic cells that are constantly surveying for conserved pathogen associated molecular patterns (PAMPs) derived from microbes that breach initial physical barriers. Various TLR agonists have been studied as potential adjuvants, including porin B (PorB) for TLR2, poly(I:C) for TLR3, and CpG for TLR9, with studies showing enhanced immunity against a number of antigens (J.-K. Li et al. 2017; Tomai and

Vasilakos 2012; Bhardwaj, Gnjatic, and Sawhney 2010). Unmethylated CpG DNA is recognized by TLR9, and activates the innate arm of the immune system, which can help drive a Type 1 T helper cell (Th1) response, critical for cellular mediated immunity. Studies have tested CpG as a potential mucosal adjuvant, where the plasmacytoid dendritic cells (pDC) that they act on are present in the mucosa (Iho, Maeyama, and Suzuki 2015). This represents an attractive target given that the mucosa is a point of entry for many viral infections. However, much work is being done to explore CpG as an adjuvant given that constant activation of TLR9 is thought to induce autoimmunity in mice.

Costimulatory molecules, including 4-1BB and ICOS, have also been utilized as adjuvants for vaccination. T cells need two signals in order to become activated: signal one, which is antigen specific and involves the interaction between the T cell receptor and peptide major histocompatibility complex (MHC), and signal two which is antigen nonspecific and involves the interaction between costimulatory molecules on the APC and T cell. Without this second signal, T cells will become anergized and will no longer become activated, even if they come into contact with the antigen that they are specific for. The addition of these costimulatory molecules as adjuvants to DNA vaccines represent an opportunity to enhance the interaction between the APC and T cell, and potentially impact the magnitude of immune response. In a non-human primate (NHP) simian immunodeficiency virus (SIV) study, macaques adjuvanted with 4-1BB showed enhanced $CD8^+$ T cell responses compared to DNA vaccine only (Hokey et al. 2007; Hirao et al. 2011).

Chemokines, including CCL7, CCL4, and CCL19, are attractive as potential adjuvants due to their inherent properties as chemotactic agents. In a DNA vaccine setting, these agents may be able to draw in different cell populations that can expand the immune response to the delivered agent. CCL4 has been shown to recruit dendritic cells, which may ultimately increase antigen presentation, as well as natural killer cells and monocytes. CCL4 was able to adjuvant a DNA

vaccine in a cancer mouse model, and partially ameliorate tumor burden (Nguyen-Hoai et al. 2016).

Cytokines, which are group of secreted proteins that mediate cross-talk between cells during immune responses among other functions, have been widely explored as adjuvants for vaccines. They play a critical role in inflammatory responses, and can both mediate or alleviate inflammation depending on the class that they fall under. Proinflammatory cytokines include IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, TNF-α, and IFN-γ. Anti-inflammatory cytokines include IL-37 and IL-38. Cytokines have been known to act as growth factors, mediate cellular responses, drive antibody responses, and alarm the immune system of pathogens. Granulocyte-macrophage colony-stimulating factor (GM-CSF), a white blood cell growth factor with strong adjuvant properties, was one of the first cytokines that demonstrated a clear impact on DNA vaccine-induced immunity. Mice immunized with GM-CSF along with a rabies DNA vaccine had increased antibody production, stronger $CD4^+$ T cell responses and were protected after a lethal challenge (Zhou et al. 2013).

IL-12 is a strong Th1 adjuvant that can expand $CD8^+$ and $CD4^+$T cell responses. It has garnered much attention in the field for its adjuvant properties in a number of preclinical models. In a non-human primate (NHP) study where monkeys were given an HIV-1 DNA vaccine and challenged, primates adjuvanted with IL-12 showed increased cellular responses that corresponded with better viremia control (Chong et al. 2007). Data from a clinical study showed that the inclusion of plasmid IL-12 as part of an HIV synthetic DNA vaccine increased T cell magnitude and response rates in people (Kalams et al. 2013).

Members of the IL-1 superfamily, the IL-36 family is made up of pro-inflammatory mediators alpha, beta, gamma, as well as antagonist IL-36Ra (Catalan-Dibene, McIntyre, and Zlotnik 2018; Clavel, Thiolat, and Boissier 2013; Dinarello 2013). This relatively novel cytokine family remains poorly understood, although recent important studies have begun to shed light on their

mechanism of action. Upon binding to the IL-36 receptor (IL-36R), and recruitment of the co-receptor accessory protein (IL-1RAcP), these cytokines activate the NF-κB/MAPK pathway resulting in the stimulation of pro-inflammatory intracellular responses, whereas binding of the antagonist, IL-36Ra, prevents recruitment of IL-1RAcP and does not lead to intracellular response. IL-36R is primarily expressed on naïve $CD4^+$ T cells, but is also found on dendritic cells, while the cytokines are expressed mainly in skin keratinocytes and epithelium, although they are also expressed at low levels in the lung, kidneys, and intestine. Given reports of IL-36 beta's ability to amplify Th1 responses (Vigne et al. 2012; 2011; Dietrich et al. 2016), we sought to understand whether these cytokines could act as adjuvants for DNA vaccination models. Here, we describe that a novel designed truncated IL-36 gamma (opt-36γt) as a co-formulated adjuvant plasmid, boosts humoral as well as $CD4^+$ and $CD8^+$ T cell immunity against three model synthetic DNA antigens including HIV Env, Influenza H1, and ZIKV-prME. Furthermore, opt-36γt enhanced protection by improving both clinical symptoms and mortality against a Zika virus challenge and provided significant dose sparing for the Zika vaccine as studied using a suboptimal vaccine dose model. This not only supports the potential of opt-36γt as a gene adjuvant, but also highlights an underappreciated area of importance for protective cellular immune responses in Zika virus pathogenesis. Further investigation into opt-36γt as a potential new adjuvant for enhancing immunity against vaccine antigens is warranted.

Results

Truncation of IL-36 beta enhances immune responses against HIV DNA vaccine. While the IL-36 family was discovered in 1999, members of this family remain poorly understood and continue to be investigated. In the initial studies of their biology, large quantities of IL-36 ligands were needed, in greater excess than those traditionally used for cytokines, to observe their activity. Given the fact that many of the IL-1 family members including IL-1α, IL-1β, IL-18, and IL-33 require proteolytic cleavage to gain activity, it was not altogether surprising that recent

reports demonstrated the need for N-terminal residue truncation to achieve full bioactivity of IL-36 ligands (Towne et al. 2011). The lack of caspase cleavage motifs in the N-terminus of the cytokines has confounded the field, however recent studies suggested the role of neutrophil proteases in activating IL-36 cytokines (Henry et al. 2016). In our studies, we examined whether truncation was important for an IL-36 *in vivo* produced gene adjuvant to impact immune profile of DNA vaccine antigens in an *in vivo* DNA vaccine model system (Figure 3). We chose to initially start our studies with IL-36 beta, as IL-36 beta has been reported to amplify Th1 responses, making it a potential cellular adjuvant candidate. We designed two DNA constructs encoding either full length (opt-36β) or truncated (opt-36βt) IL-36 beta for these comparative studies. We added a highly efficient IgE leader sequence to both of the sequences as well as RNA and codon optimized them in order to enhance protein expression (Figure 4). We then immunized C57BL/6 (B6) (n=5) mice with 2.5 μg of HIV Env DNA alone or with 11 μg opt-36β or opt-36βt, three times at three-week intervals using the 3P electrode driven by an adaptive electroporation CELLECTRA (EP) device. Spleens were harvested ten days post-final vaccination for analysis of antigen specific responses (Figure 5). We observed a significant increase in the number of antigen specific $CD4^+$ T cells that secreted IFN-γ and TNF-α in the animals whose vaccine included opt-36βt compared to opt-36β (Figure 6). There was a trend towards a similar pattern of enhancement for the antigen induced $CD8^+$ T cell responses, but in contrast to the $CD4^+$ T cell responses, this did not reach significance. A dosing study was next performed, focusing primarily on T cell induction to determine the optimal dose of opt-36βt (Figure 7). We found no significant difference in T cell response with higher doses and, in fact, there appeared to be a trend towards decreased immune response at the 30μg dose of opt-36βt. Going forward, we maintained our established dose of 11μg dose for adjuvant plasmid for the remainder of the studies.

Expression of all three truncated IL-36 cytokines. Given the immune response enhancement observed with truncation of IL-36 beta, we next examined the rest of the IL-36 family as truncated

cytokines. In this regard, even less is known about IL-36 alpha or gamma compared to beta, so we wanted to evaluate the immune responses in mice adjuvanted with each of the three cytokines in comparative studies. Truncated IL-36 alpha (opt-36αt) and IL-36 gamma (opt-36γt) were designed and modified as described for opt-36βt (Figure 8). Construct expression *in vitro* was confirmed by Western blot and IFA (Figures 9 and 10).

Opt-36βt and opt-36γt maintain vaccine-induced responses at a memory time point. A major concern in the vaccine field is the generation of candidates that can provide durable, long-term immune responses. Generally, people are immunized to prevent future illness, and so special consideration must be taken into account for the durability of induced immune responses. With this in mind, we examined whether immune responses following DNA vaccination would be maintained into memory. B6 mice (n=5/group) were immunized using 2.5 μg of HIV Env DNA alone or formulated with 11 μg of opt-36αt, opt-36βt, or opt-36γt three times at three-week intervals with CELLECTRA 3P electroporation (EP). Spleens were harvested 50 days post-final vaccination to analyze antigen specific responses at a memory time point (Figure 11). A quantitative ELISpot was performed to determine the number of Env specific IFN-γ secreting T cells that responded to vaccination. We observed that mice immunized with the HIV vaccine alone produced an average of 775 spot forming units (SFU)/million splenocytes, while mice adjuvanted with opt-36αt, opt-36βt, and opt-36γt had an average of 1242, 1460, and 1610 SFU/million splenocytes, respectively, supporting a potently enhanced cellular response to the vaccine was driven by the adjuvants. Similar to the results observed at an acute time point, we found that mice adjuvanted with opt-36βt showed a significant increase in the percent of $CD4^+$ T cells that expressed IFN-γ and TNF-α, compared to vaccine only (Figure 12). Interestingly, mice adjuvanted with opt-36γt showed a 3-fold enhancement in the percent of vaccine specific $CD8^+$ T cells, which expressed IFN-γ and TNF-α. We further observed that mice vaccinated with vaccine

and opt-36γt had a significant increase in the percent of $CD107a^+$ IFN-γ^+ $CD8^+$ T cells, suggesting the cytolytic potential of these cells (Figure 13).

Opt-36γt boosts humoral responses in an influenza DNA vaccine model. We next sought to extend this finding to additional DNA vaccine antigens. We studied opt-36αt, opt-36βt, and opt-36γt's ability to impact immune responses driven by an HA1 influenza DNA vaccine. Given the potency of the adjuvant response in the previous HIV Env studies, we focused on a two-dose regimen to evaluate the vaccine-induced immune response in a dose-sparing model. Balb/C mice (n=5/group) were immunized two times at two-week intervals with either 1 μg of HA1 DNA alone or 1 μg of HA1 and 11 μg of opt-36αt, opt-36βt, or opt-36γt followed by *in vivo* EP. We observed that both opt-36βt and opt-36γt significantly enhanced cellular responses compared to the low dose vaccine alone, 10 days post final immunization (Figure 14). We observed increased cellular responses in mice adjuvanted with opt-36αt, however this was not as pronounced as the responses seen with the other two ligands. As antibodies are known to be critical for prevention of influenza infection, we studied the binding antibody response generated post vaccination. Opt-36γt elicited significant higher endpoint binding titers compared naïve mice (Figure 15). We further examined the quality of these antibodies, by performing an ELISA based avidity test to examine strength of binding to a HA1 influenza protein (Figure 16). Interestingly, we observed that only mice adjuvanted with opt-36γt had higher antibody binding and maintained avidity while opt-36αt adjuvanted mice had lower antibody binding and avidity compared to vaccine alone and opt-36βt adjuvanted mice had higher antibody binding but lower antibody avidity. These data support the induction of improved humoral responses by opt-36γt, and potentially suggest a role for T follicular helper cells (T_{fh}), which are important for refining the antibody response over time. We also examined the isotypes of the antibodies generated post vaccination in order to assess potential Fc receptor mediated immune response such as antibody dependent cellular cytotoxicity (ADCC), but did not observe significant isotype switching between the groups (Figure 17).

Opt-36γt improves cellular responses induced by a nonprotective dose of Zika DNA vaccine. Based on the data generated in the two DNA vaccine models above, we focused on studying opt-36γt in combination with a DNA vaccine against Zika virus and observing how adjuvanted vaccine-induced immune response impacted challenge outcome. This model allowed us to confirm the relevance of the improved immunity and dose sparing potential driven by the opt-36γt adjuvant. Previous murine studies have described a protective dose as 25 μg, however a lower dose is necessary to observe adjuvant impact. We immunized IFNAR $^{-/-}$ mice (n = 5-6 mice/group) once with an exceptionally low dose (0.5 μg) of ZIKV-prME DNA vaccine alone or a combination of vaccine and opt-36γt. Two weeks following vaccination, we harvested spleens and blood. We observed that mice immunized with low dose vaccine only did not generate significant IFN-γ ELISpot responses, but the combination of the vaccine and opt-36γt drove cellular response, resulting in 700 SFU/million splenocytes, similar to the magnitude of response observed in previous studies when mice are immunized with 25 μg of ZIKV-prME vaccine alone (Figure 18). Through intracellular cytokine staining measurements, we observed that mice adjuvanted with opt-36γt exhibited increased IFN-γ and TNF-α expressing $CD4^+$ T cells as well as IFN-γ expressing $CD8^+$ T cells compared to the vaccine only treated mice (Figure 19). Overall antibody responses were very low in all groups, suggesting a need for additional vaccine boosts or higher vaccine doses to further characterize the humoral immunity induced in this model (Figure 20).

Opt-36γt codelivery protects mice against Zika challenge. In order to assess whether the enhanced immune responses observed were protective, we repeated the study and this time performed a challenge using IFNAR $^{-/-}$ mice (n = 12-14/ group) with a lethal dose of a validated Zika virus stock, strain PR 209. Challenge was performed two weeks after an immunization with either 0.5 μg of ZIKV-prME alone or in combination with 11 μg of opt-36γt. The animals were followed for two weeks post challenge. One of the main side effects of Zika challenge typically

observed in this mouse strain is weight loss. Significant weight loss was observed in both the naïve and mice immunized with the suboptimal dose of the ZIKV-prME vaccine alone demonstrating substantial morbidity from the challenge (Figure 21). Naïve mice appeared to be the most impacted, with many mice losing up to 20% of their starting body weight. The low dose vaccine only group fared a bit better compared to the naive but still lost a considerable amount of weight. Strikingly, mice immunized with ZIKV-prME in combination with opt-36γt were protected against weight loss, gaining weight during the course of the study. Additionally, mice were monitored for clinical symptoms during the challenge (Figure 22). Mice in both naïve and vaccine only groups became progressively sicker (i.e. hunched posture and paralysis of hind limbs) between days 5 and 7. However, the adjuvanted mice remained healthy and showed no sign of disease following challenge. As animals succumb to disease they are sacrificed at predefined humane endpoints. Dramatically, mice immunized with ZIKV-prME and opt-36γt exhibited a robust 92% survival rate, compared to 28% for mice immunized with the ZIKV-prME only and 13% for naïve mice (Figure 23), illustrating the significant benefit of the opt-36γt adjuvant in the context of this challenge model.

Discussion

While the IL-36 cytokine family was first discovered nearly two decades ago, it is only recently that roles for these cytokines are beginning to be elucidated. The IL-36 family, members of a larger proinflammatory IL-1 family, has been primarily implicated for their potential role in pustular psoriasis and inflammation of the skin and joints (Johnston et al. 2017; Ding et al. 2017; Foster et al. 2014; Kanazawa et al. 2013). Dysregulation of the natural IL-36 receptor antagonist or overexpression of IL-36 in the skin has been implicated in a number of skin diseases and conditions. However, some of these proinflammatory properties have also piqued the scientific community's interest regarding some of the other roles that these cytokines might play. Following

reports that IL-36 beta could amplify Th1 responses in $CD4^+$ T cells, a number of studies have shown the induction of IL-36 cytokine expression, especially IL-36 gamma, in response to infections including pneumonia, herpes simplex virus (HSV), and candidiasis, suggesting that IL-36 cytokines may play a significant role in host immunity (Verma et al. 2018; P. Wang, Gamero, and Jensen 2019; Winkle, Throop, and Herbst-Kralovetz 2016; X. Wang et al. 2015a; Kovach et al. 2017).

To our knowledge, this is the first study to compare the effects of all three truncated IL-36 cytokines in a vaccination model. In these studies we provide additional insight into the ability of truncated IL-36 gamma's (opt-36γt) ability to boost immune responses using three DNA vaccine antigens. As previously demonstrated by Towne *et. al*, we found that truncation of the IL-36 cytokines nine amino acids at the N-terminal region was critical for their activity to enhance vaccine-induced immune responses. For future investigations of IL-36 cytokines in protective immunity studies, the truncated forms of these cytokines will almost certainly be necessary to exploit their full potential.

In the DNA vaccine models we tested, we found that mice immunized with opt-36βt and opt-36γt were both able to enhance vaccine-induced cellular immune responses. However, where opt-36βt was able to significantly increase the number of antigen-specific IFN-γ^+ and TNF-α^+ $CD4^+$ T cells, opt-36γt significantly increased the number of antigen-specific IFN-γ^+, TNF-α^+, and $CD107a^+$ $CD8^+$ T cells, suggesting an impact of opt-36γt to improve cytolytic activity of these cells. Further work must be done to understand the differences between the two cytokines' seemingly preferential action on various cell compartments. Regarding humoral immunity in this model, we found that opt-36γt was able to increase antibody-binding titers, while opt-36βt appeared to either suppress the antibody response or induce antibodies that have weak avidity. Thus, in our models, opt-36γt can improve both arms of immune response, which is likely important for many of the challenging disease targets that remain. Furthermore, we found that the synergy of a non-

protective dose of Zika DNA vaccine with opt-36γt was able to protect mice against a lethal Zika challenge, highlighting the potential of opt-36γt to affect challenge outcome and drive protection. A caveat to our studies on opt-36γt is that although opt-36γt was able to enhance antibody binding in the HIV and influenza DNA models, overall humoral responses in the Zika DNA model were very low. This suggests that there may be a minimum dose of vaccine required to enhance antibody responses and/or that there may be differential activities of these cytokines depending on the antigen, and further work to investigate these conditions are underway. Although the antibodies generated after vaccination were low, the addition of opt-36γt was still able to protect against Zika challenge, highlighting the importance of cellular immunity in this model, even in the absence of a robust antibody response.

There is still much work to be done to fully understand the roles that the IL-36 cytokines play under both homeostatic and pathologic conditions in the host immune system. Multiple studies in mice have shown that the IL-36 cytokines may have distinct functions in response to different inflammatory stimuli. Understanding how opt-36βt and opt-36γt may exert their activities on different cell populations and against additional vaccine targets will be important for further harnessing their potential. Given their ability to enhance $CD4^+$ and $CD8^+$ T cell responses, opt-36γt and opt-36βt look especially promising for disease models in which cellular responses are important, such as cancer where driving $CD8^+$ immunity is important to clear tumors. Studies examining the effects of opt-36γt on driving tumor-infiltrating lymphocytes (TILS) would be relevant. Work by Wang *et al* has demonstrated that tumor growth was significantly inhibited in B16 melanoma IL-36 expressing cells compared to control B16 cells that did not express IL-36 gamma in mice (X. Wang et al. 2015b). Wang et al. also found that IL-36 gamma could promote early activation and expansion of naïve $CD8^+$ T cells, in line with what we have observed in our DNA vaccine models.

The induction of higher binding antibodies while maintaining avidity by opt-36γt as seen in the influenza studies may have a critical role in diseases in which highly refined and mature antibodies are important. As more emphasis is being focused to identify immunogens that can elicit broadly neutralizing antibodies (bNabs) for HIV and Influenza, adjuvants that can further refine the antibody response may prove important. Future studies that hone in on the effects of IL-36 cytokines, especially IL-36 gamma, on the T cell populations that support enhanced antibody affinity such as T follicular helper cells (T_{fh}), will help to further characterize the immune responses elicited during vaccination, and also deepen the understanding of the IL-36 cytokine family as well. In addition, deeper characterization of the antibodies generated with IL-36 adjuvanted vaccination, such as complementarity-determining region (CDR) sequencing, especially for the CDR3 region where the most sequence variability exists, will likely further broaden the understanding of these cytokines' activities, especially for IL-36 gamma.

Although there appears to be a deleterious effect on skin health when IL-36 signaling is left unchecked, localized controlled and likely transient delivery of opt-36γt as an adjuvant during intradermal vaccination could enhance vaccine responses and recruitment of cells to the site of infection. This could be especially important for infectious diseases that breach the skin's natural barrier including herpes, malaria, and Leishmania among others. As the largest organ in the human body, with a rich source of antigen presenting cells (APCs) including Langerhans cells, as well as nearly 20 billion T cells, the skin is a particularly attractive site to administer an opt-36γt adjuvanted vaccine. Enhanced CTL responses in the skin can help control the spread of an infection before it is able to disseminate to other locations in the body, while greater antibody responses may help with prevention of infection. Studies that examine the delivery of opt-36γt in the skin compared to intramuscular delivery may shed light on another route to impact vaccine immune outcome as well as protection against infection.

As the global population and the demand for vaccines increase worldwide, the need to maximize immune responses while minimizing the effective dose necessary to induce protective responses will continue to grow to further control costs. Opt-36γt, which has shown adjuvant activity as a dose sparing adjuvant, may represent a potential avenue to meet some of these demands. The work presented in these studies in addition to all the amazing work being done in the vaccine field has reinforced the need for more focus on developing new adjuvants that work in a variety of settings for other vaccine platforms and generating their adjuvant effects through unique mechanisms of action.

*Modified from: Louis L, Wise MC, Choi H, Muthumani K, Villarreal DO, Weiner DB. Designed DNA encoded IL-36 gamma acts as a potent molecular adjuvant enhancing Zika synthetic DNA vaccine induced immunity and protection in a lethal challenge model. *Vaccines.* 2019; 7(2) 42

MMAFPPQSCVHVLPPKSIQMWEPNHNTMHGSSQSPRNYRSSQSPRNYR**VHD**S
QQMVWVLTGNTLTAVPASNNVKPVILSLIACRDTEFQDVKKGNLVFLGIKNRNLCF
CCVEMEGKPTLQLKEVDIMNLYKERKAQKAFLFYHGIEGSTSVFQSVLYPGWFIAT
SSIERQTIILTHQRGKLVNTNFYIESEK

MSSQSPRNYR**VHD**SQQMVWVLTGNTLTAVPASNNVKPVILSLIACRDTEFQDVKK
GNLVFLGIKNRNLCFCCVEMEGKPTLQLKEVDIMNLYKERKAQKAFLFYHGIEGST
SVFQSVLYPGWFIATSSIERQTIILTHQRGKLVNTNFYIESEK

Figure 3.Full length and truncated IL-36 beta sequences.

Full-length IL-36 beta and the truncated IL-36 beta cytokine sequence. The sequence was truncated 9 amino acids N-terminal to the structural motif highlighted in red.

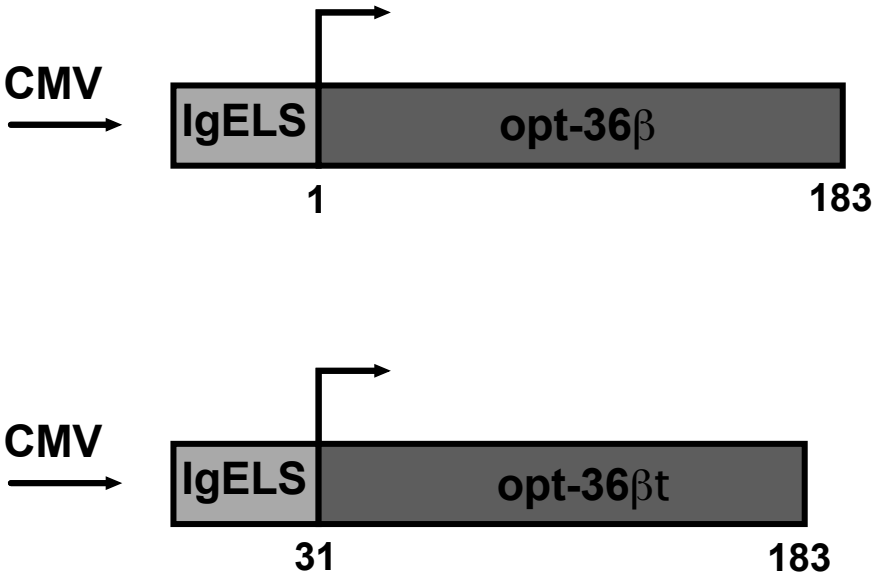


Figure 4. IL-36 beta sequence design.

These maps illustrate the design if the optimized IL-36 beta constructs. Opt-36β is the full length beta cytokine that has been RNA and codon optimized and is under the control of the immediate early cytomegalovirus (CMV) promoter. Opt36βt is the truncated form of IL-36 beta, with the first 30 amino acids removed. An efficient IgE leader sequence was added to the beginning of both sequences, and both sequences were inserted into a pVax backbone.

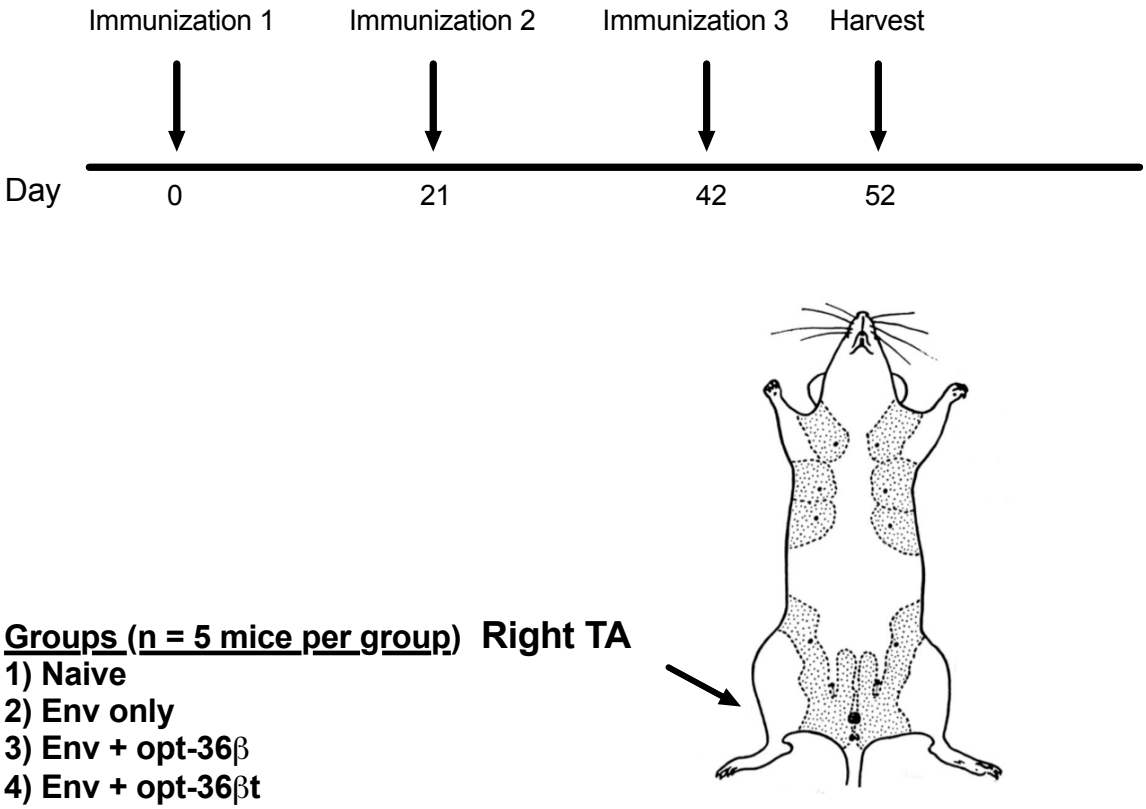


Figure 5. Delivery of full length vs truncated IL-36 beta.

C57BL/6 mice (n= 5 mice/group) received an intramuscular DNA injection and EP of either 2.5 µg of HIV Env vaccine alone, 2.5 µg of HIV Env vaccine and 11µg of opt-36β (full length optimized IL-36 beta), or 2.5 µg of HIV Env vaccine and 11µg of opt-36βt (truncated optimized IL-36 beta), three times at three week intervals in the tibialis anterior muscle. Spleen and blood were harvested 10 days after the final immunization.

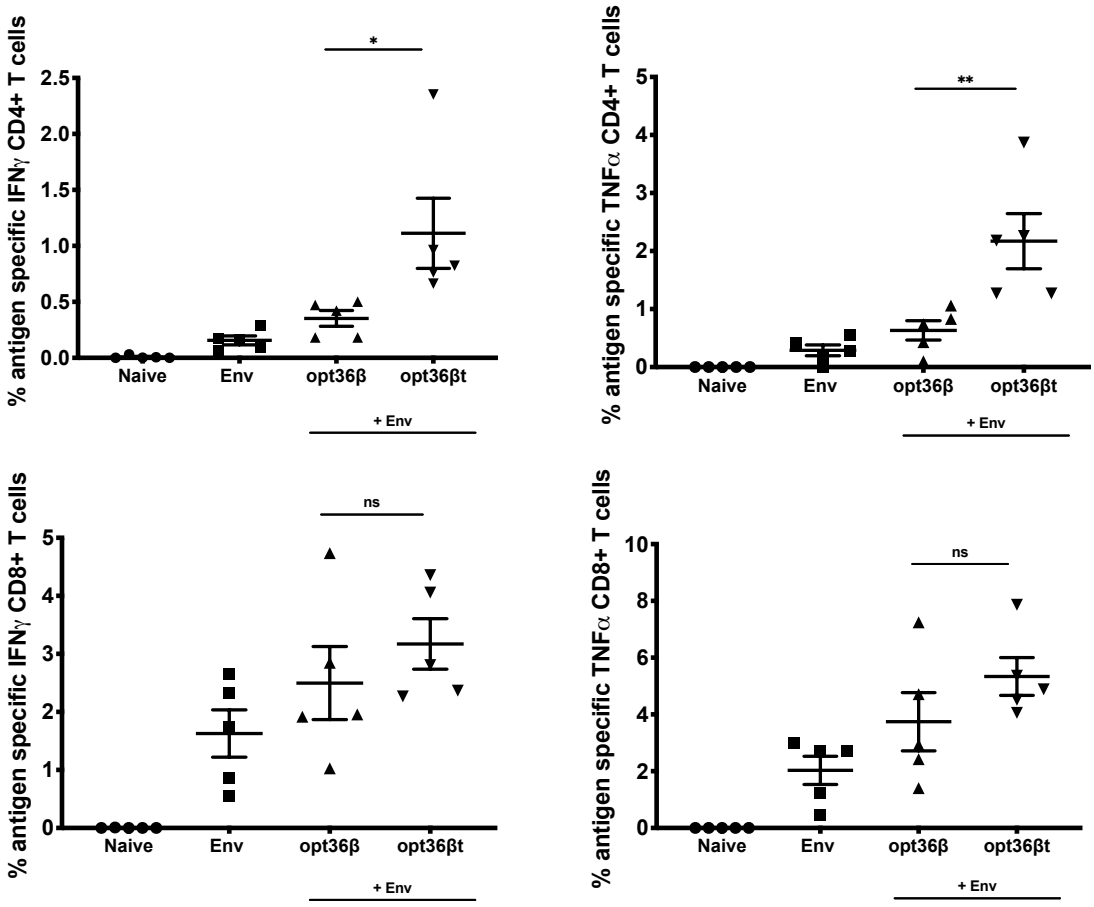


Figure 6. Vaccine induced CD4$^+$ and CD8$^+$ IFN-γ and TNF-α T cell responses post opt-36β or opt-36βt codelivery

C57BL/6 mice (n = 5 mice/group) received an intramuscular DNA injection and EP of either 2.5 μg of HIV Env vaccine alone, 2.5 μg of HIV Env vaccine and 11μg of opt-36β (full length optimized IL-36 beta), or 2.5 μg of HIV Env vaccine and 11μg of opt-36βt (truncated optimized IL-36 beta), three times at three week intervals in the tibialis anterior muscle. Antigen specific CD4$^+$ and CD8$^+$ T cell responses measured by intracellular cytokine staining 10 days after final immunization. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

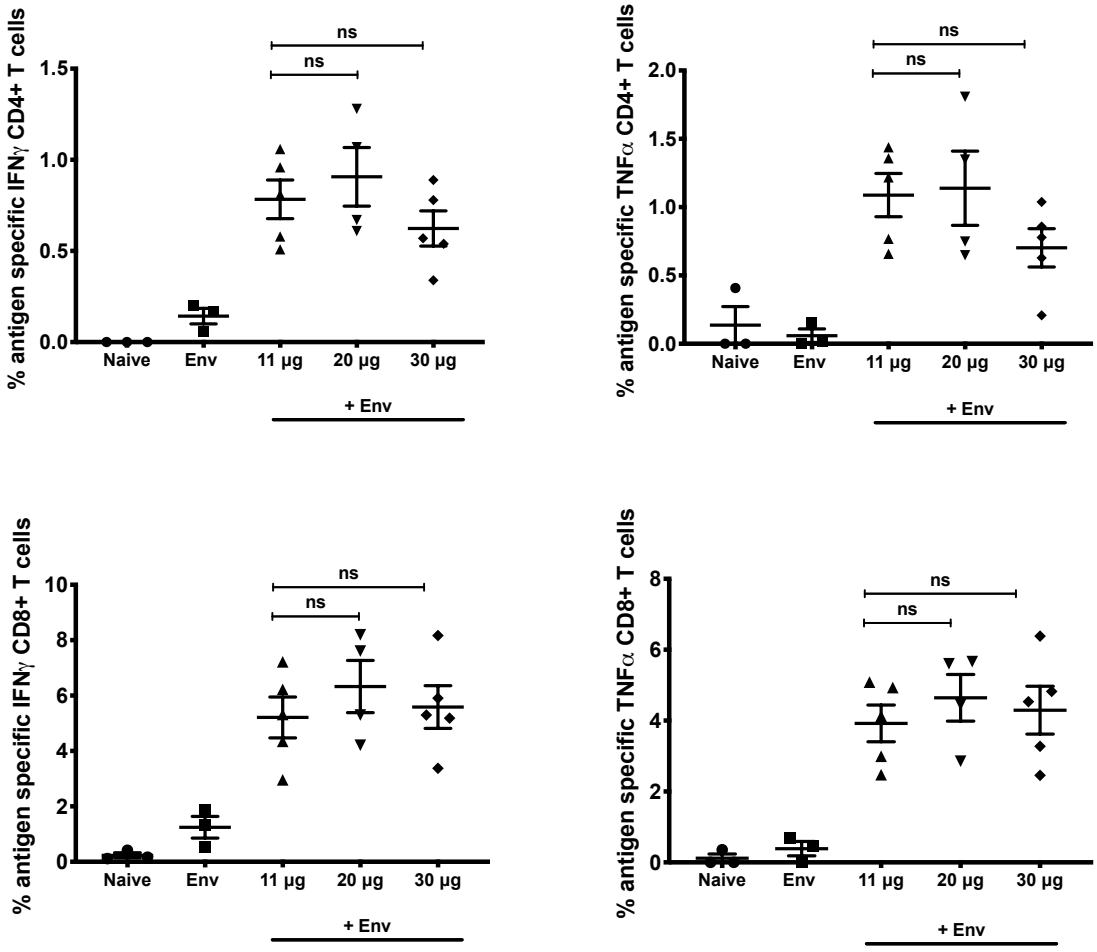


Figure 7. Vaccine induced $CD4^+$ and $CD8^+$ IFN-γ and TNF-α T cell responses post dosing study of opt-36βt

C57BL/6 mice (n = 5 mice/group) received an intramuscular DNA injection and EP of either 2.5 µg of HIV Env vaccine alone, 2.5 µg of HIV Env vaccine and 11µg of opt-36βt (truncated optimized IL-36 beta), 2.5 µg of HIV Env vaccine and 20µg of opt-36βt, or 2.5 µg of HIV Env vaccine and 30µg of opt-36βt three times at three week intervals in the tibialis anterior muscle. Antigen specific $CD4^+$ and $CD8^+$ T cell responses measured by intracellular cytokine staining 10 days after final immunization. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

MRAASPSLRH**VQD**LSSRVWILQNNILTAVPRKEQTVPVTITLLPCQYLDTLETNRG
DPTYMGVQRPMSCLFCTKDGEQPVLQLGEGNIMEMYNKKEPVKASLFYHKKSGT
TSTFESAAFPGWFIAVCSKGSCPLILTQELGEIFITDFEMIVVH

Opt-36αt

MGRETPDFGE**VFD**LDQQVWIFRNQALVTVPRSHRVTPVSVTILPCKYPESLEQDK
GIAIYLGIQNPDKCLFCKEVNGHPTLLLKEEKILDLYHHPEPMKPFLFYHTRTGGTS
TFESVAFPGHYIASSKTGNPIFLTSKKGEYYNINFNLDIKS

Opt-36γt

Figure 8.Truncated IL-36 alpha and gamma sequences.

The sequences full length sequences that have been truncated 9 amino-acids N-terminal to the structural motif highlighted in red.

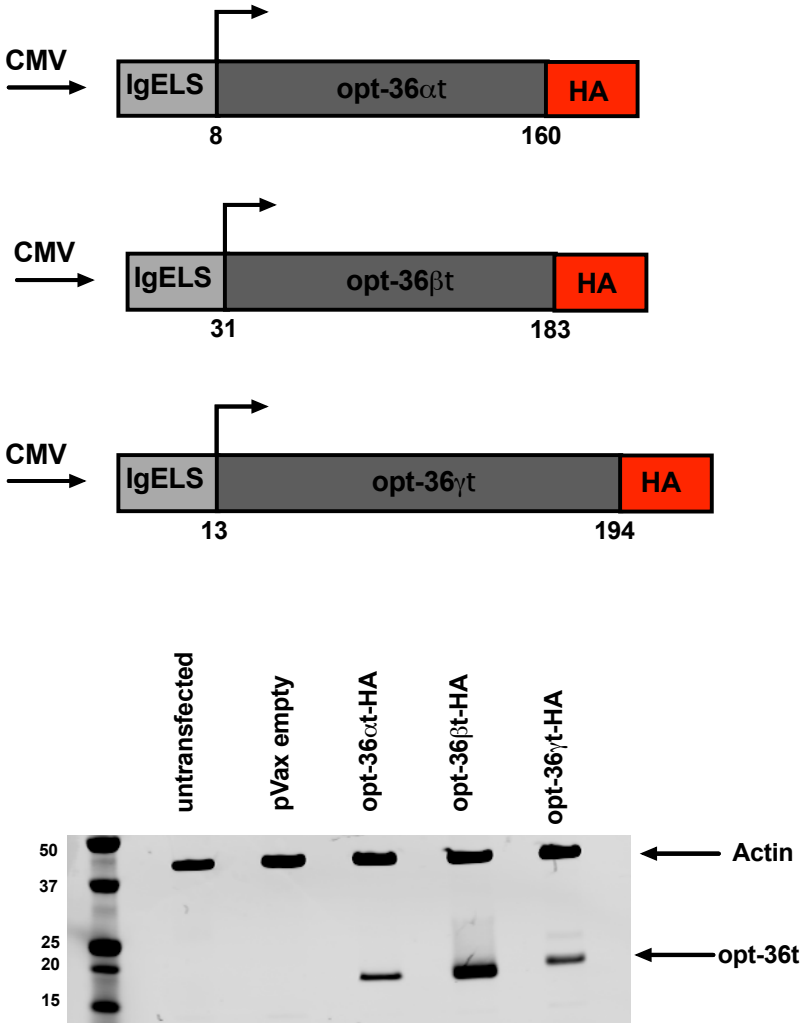


Figure 9. Optimized IL-36 cytokine constructs express *in vitro*

These maps illustrate the design of C terminus HA-tagged optimized truncated IL-36 alpha, beta, and gamma constructs. Later studies with opt-36αt, opt-36βt, and opt-36γt were performed with plasmids without an HA tag. The cytokines have been RNA and codon optimized and are under the control of the immediate early cytomegalovirus (CMV) promoter. The beginning of each sequence 9 amino acids N-terminal to a conserved anchoring motif was removed. Western blot anaylsis of opt-36αt, opt-36βt, and opt-36γt transfected U2OS lysates, separated by SDS-page under reducing conditions.

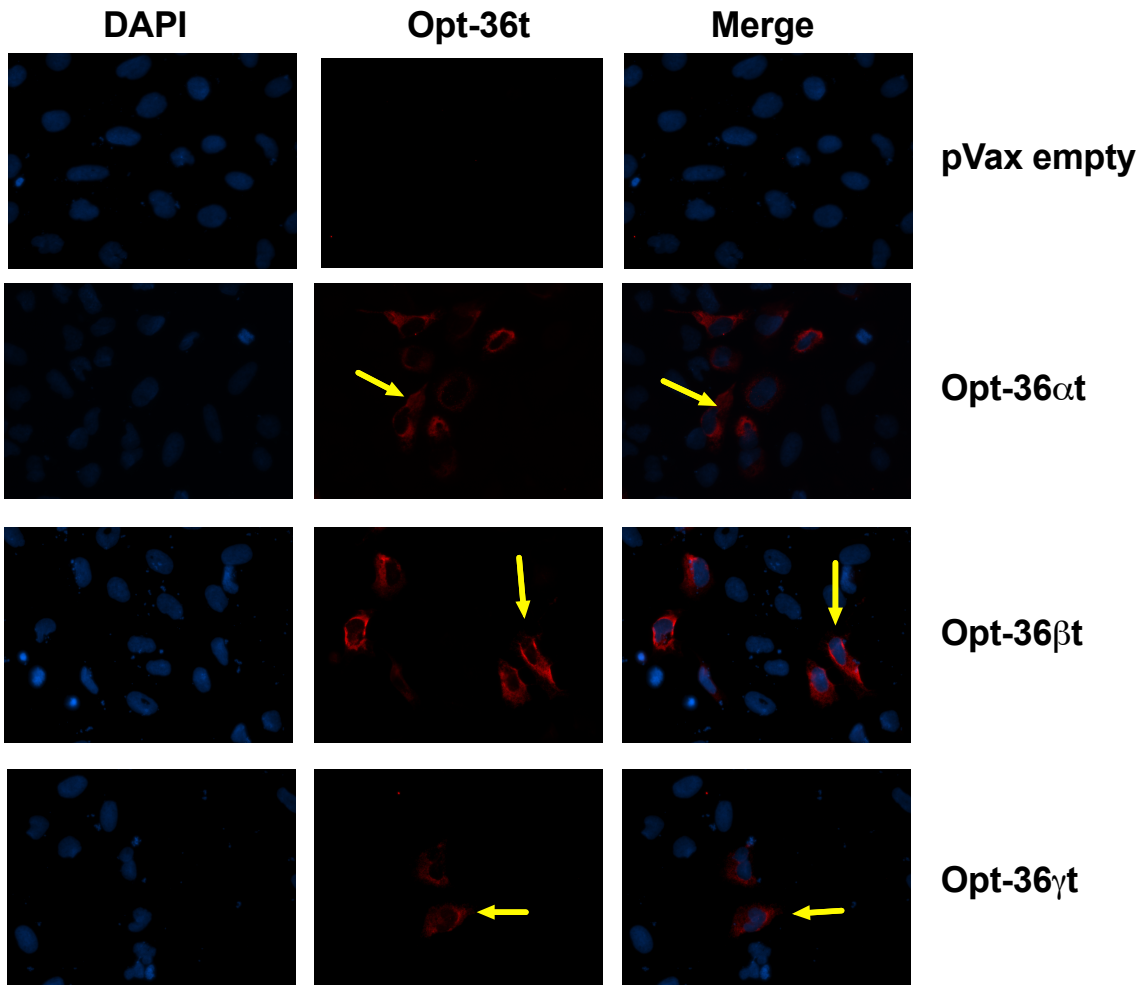


Figure 10. Immunofluorescence expression of HA-tagged opt-36αt, opt-36βt, and opt-36γt

Immunofluorescence assay (IFA) analysis of HA-tagged opt-36αt, opt-36βt, and opt-36γt transfected U2OS cells.

Groups (n = 3-5 mice per group)
1) Naive
2) Env only
3) Env + opt-36αt
4) Env + opt-36βt
5) Env + opt-36γt

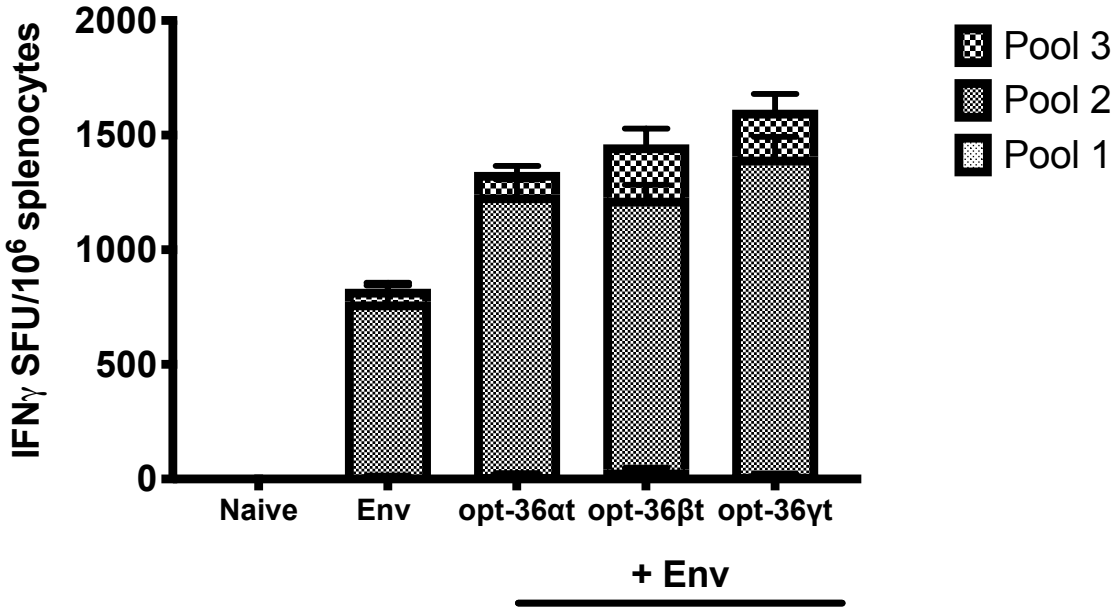


Figure 11. IFN-γ T cell response post opt-36t codelivery immunization at a memory timepoint

C57BL/6 mice (n = 3-5 mice/group) received an intramuscular DNA injection and EP of either 2.5 µg of HIV Env vaccine alone, 2.5 µg of HIV Env and 11µg of opt-36αt, 2.5 µg of HIV Env and 11µg of opt-36βt, or 2.5 µg of HIV Env and 11µg of opt-36γt three times at three week intervals in the tibialis anterior muscle. ELISpot analysis of IFN-γ responses 50 days after final immunization.

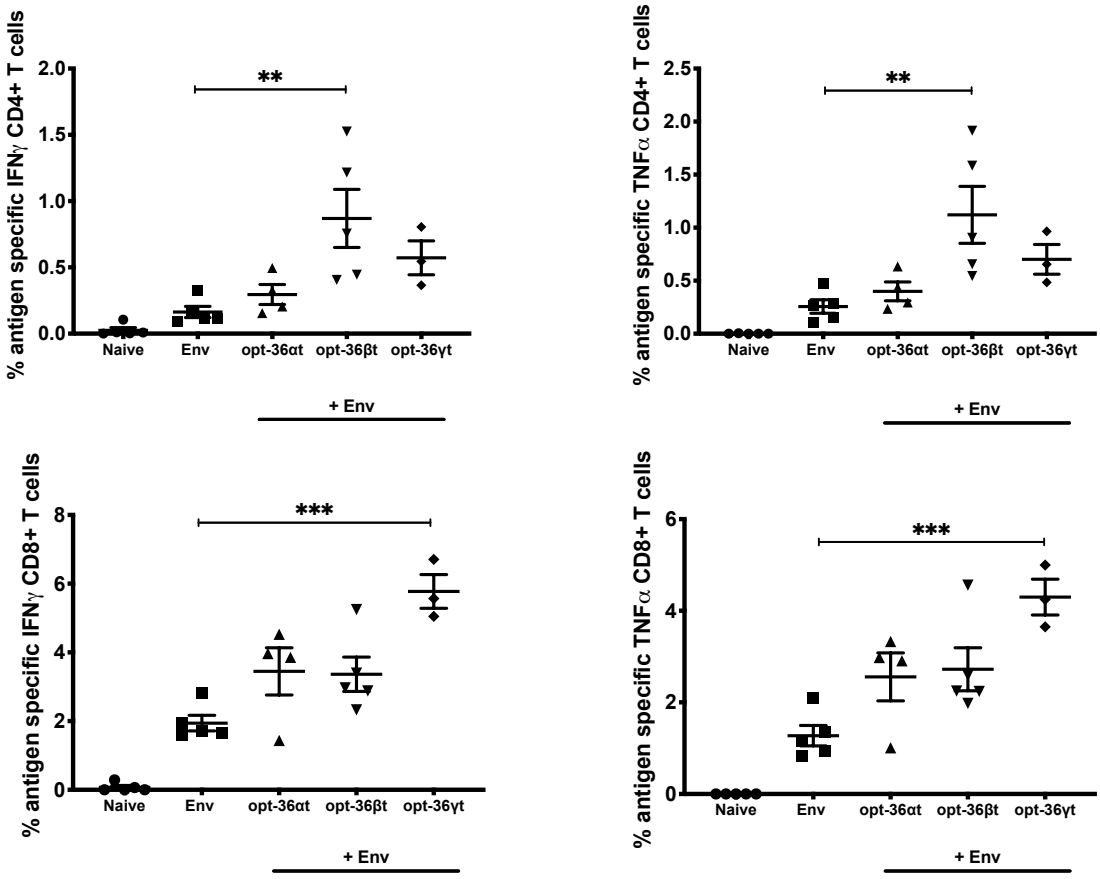


Figure 12. Vaccine induced $CD4^+$ and $CD8^+$ T cell responses at memory timepoint

C57BL/6 mice (n = 3-5 mice/group) received an intramuscular DNA injection and EP of either 2.5 µg of HIV Env vaccine alone, 2.5 µg of HIV Env and 11µg of opt-36αt, 2.5 µg of HIV Env and 11µg of opt-36βt, or 2.5 µg of HIV Env and 11µg of opt-36γt three times at three week intervals in the tibialis anterior muscle. Antigen specific $CD4^+$ and $CD8^+$ T cell responses measured by intracellular cytokine staining 50 days after final immunization. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

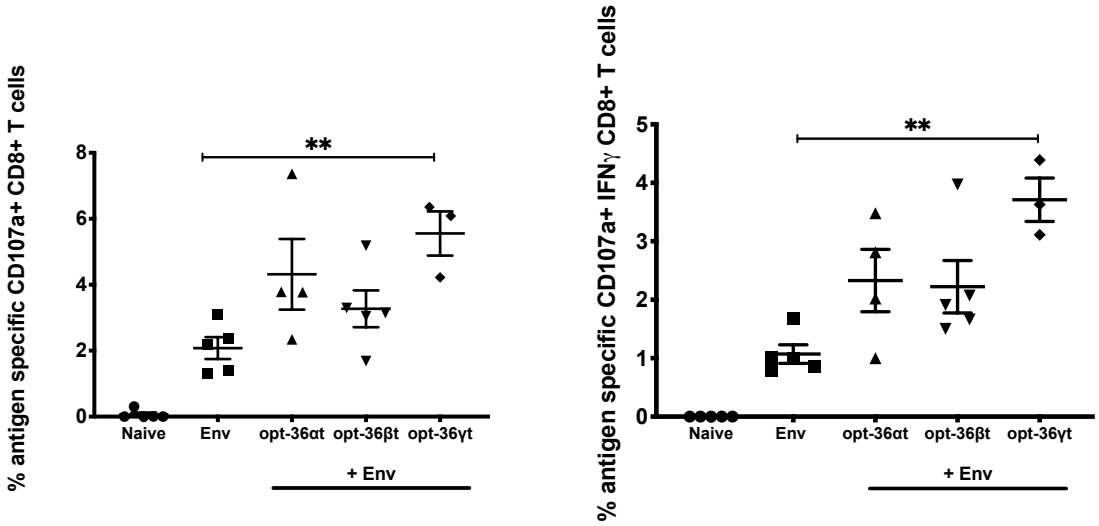
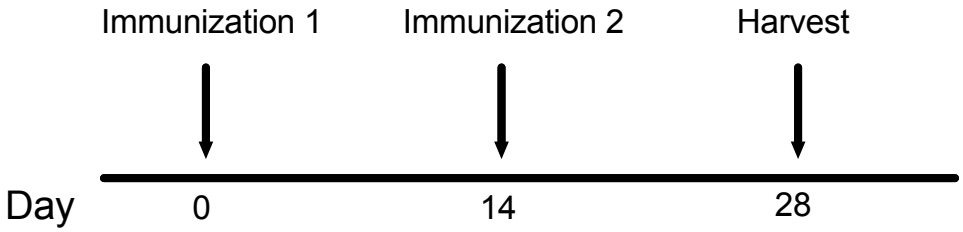


Figure 13. Vaccine induced CD107a$^+$ T cell responses at memory timepoint following opt-36t codelivery

C57BL/6 mice (n = 3-5 mice/group) received an intramuscular DNA injection and EP of either 2.5 µg of HIV Env vaccine alone, 2.5 µg of HIV Env and 11µg of opt-36αt, 2.5 µg of HIV Env and 11µg of opt-36βt, or 2.5 µg of HIV Env and 11µg of opt-36γt three times at three week intervals in the tibialis anterior muscle. Antigen specific CD107a$^+$ CD8$^+$ T cell responses measured by intracellular cytokine staining 50 days after final immunization. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$



Groups (n = 5 mice per group)
1) Naive
2) HA1 only
3) HA1 + opt-36αt
4) HA1 + opt-36βt
5) HA1+ opt-36γt

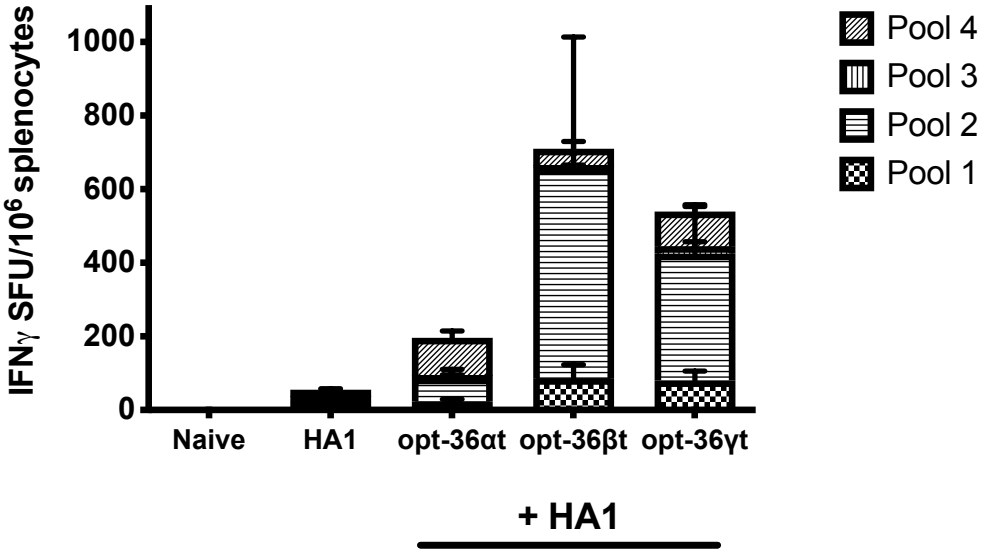


Figure 14. Vaccine induced cellular IFN-γ T cell responses following opt-36t codelivery

Balb/C mice (n = 5 mice/group) received an intramuscular DNA injection and EP of either 1µg of Influenza HA1 vaccine alone, 1 µg of Influenza HA1 and 11µg of opt-36αt, 1µg of HA1 and 11µg of opt-36βt, or 1 µg of HA1 and 11µg of opt-36γt two times at two week intervals in the tibialis anterior muscle. ELISpot analysis of IFN-γ responses 14 days after final immunization.

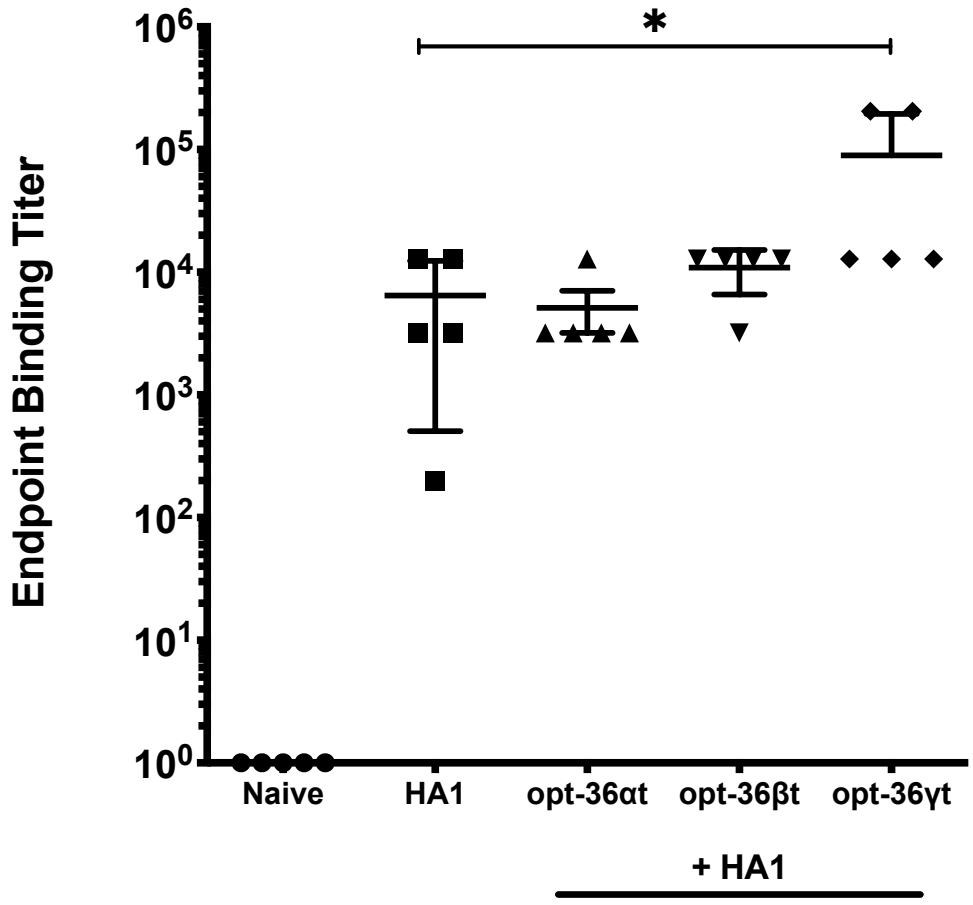


Figure 15. Opt-36γt enhances antibody response against influenza hemagglutinin protein

Antibody binding analysis of sera from mice immunized twice at two week intervals with HA1 only (1 μg), HA1 (1 μg) and opt-36αt (11 μg), HA1 (1 μg) and opt-36βt (11 μg), and HA1 (1 μg) and opt-36γt (11 μg). ELISA samples run in duplicate. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

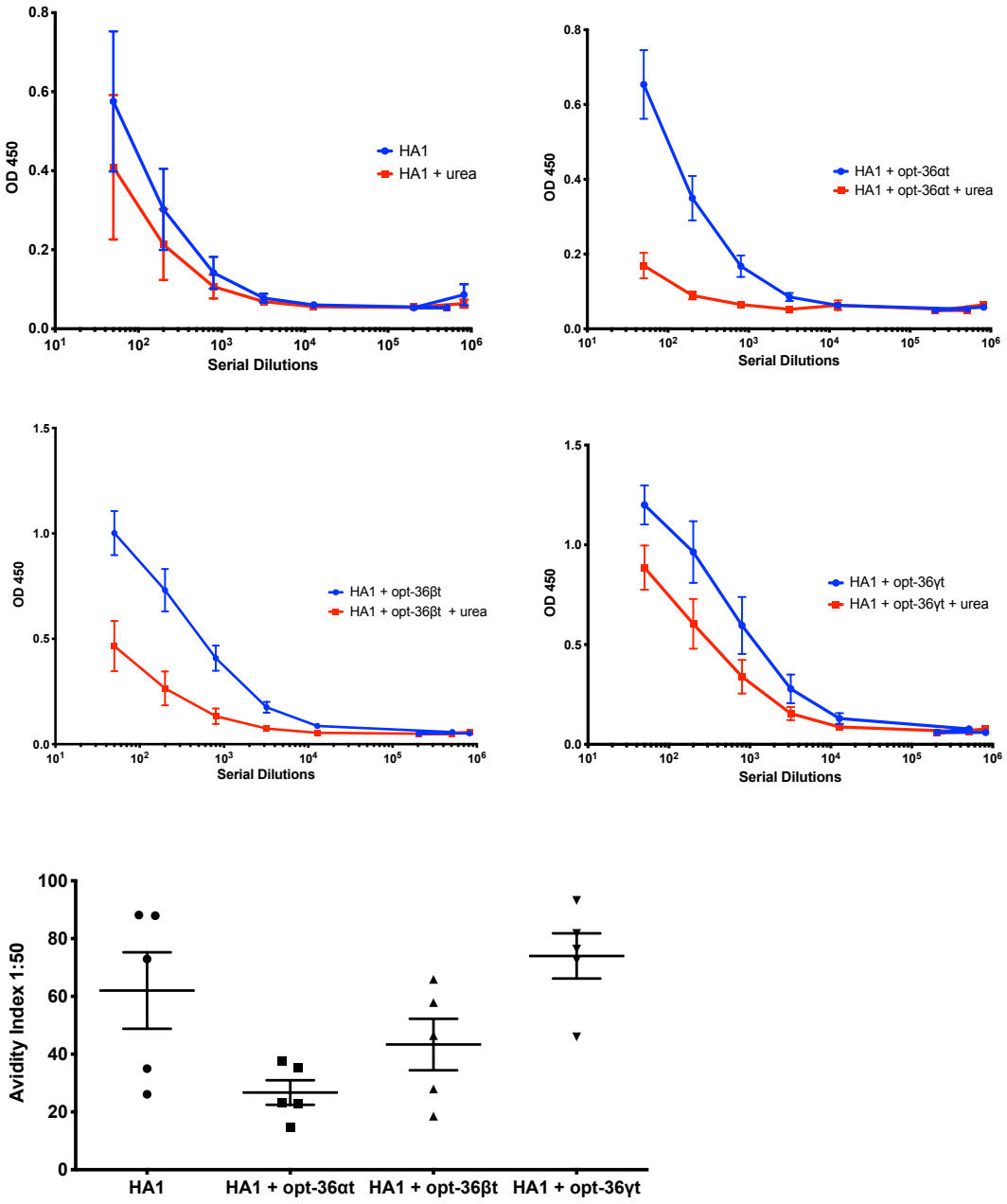


Figure 16. Opt-36γt maintains antibody avidity against influenza hemagglutinin protein after denaturing treatment

Antibody binding analysis of urea treated sera from Balb/C mice (n = 5) immunized twice at two week intervals with HA1 only (1 µg), HA1 (1 µg) and opt-36αt (11 µg), HA1 (1 µg) and opt-36βt (11 µg), and HA1 (1 µg) and opt-36γt (11 µg). ELISA samples run in duplicate. Avidity index calculated by dividing OD450 values of treated samples by OD450 values of untreated samples and multiplying by 100.

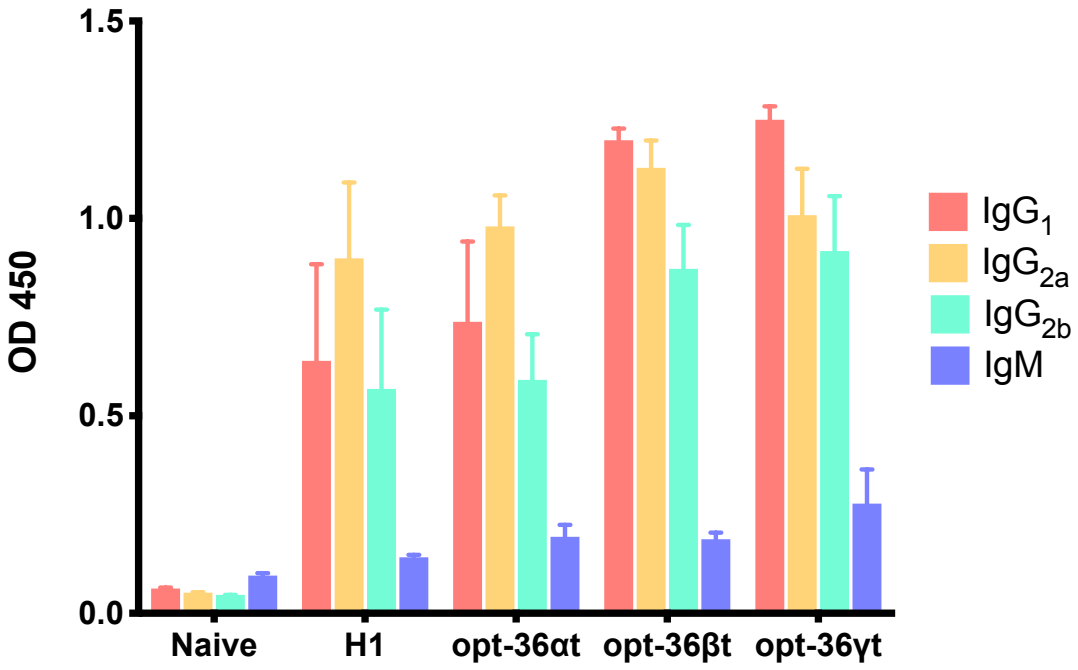
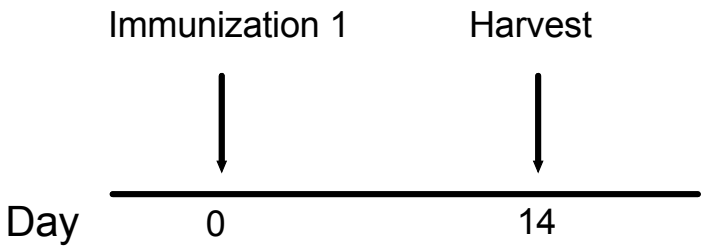


Figure 17. Antibody isotypes induced post opt36t delivery

Isotype binding analysis of sera from mice immunized twice at two week intervals with HA1 only (1 μg), HA1 (1 μg) and opt-36αt (11 μg), HA1 (1 μg) and opt-36βt (11 μg), and HA1 (1 μg) and opt-36γt (11 μg).



Groups (n = 5-6 mice per group)
1) Naive
2) prME only
3) prME + opt-36γt

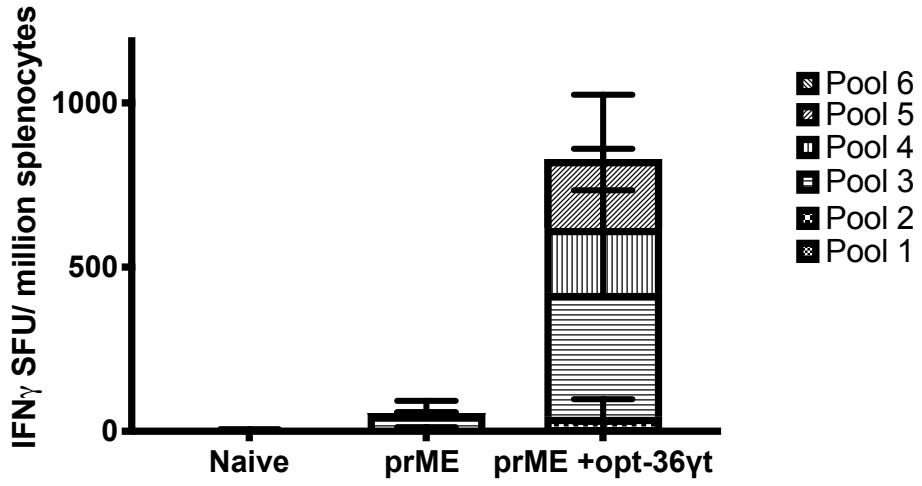


Figure 18. Opt-36γt enhances vaccine specific IFN-γ cellular responses against Zika prME vaccine

IFNAR -/- mice (n = 5-6 mice/group) were immunized once with either Zika prME vaccine alone (0.5 µg) or Zika prME (0.5 µg) and opt-36γt (11 µg). ELISpot analysis of IFN-γ cellular responses 14 days after immunization.

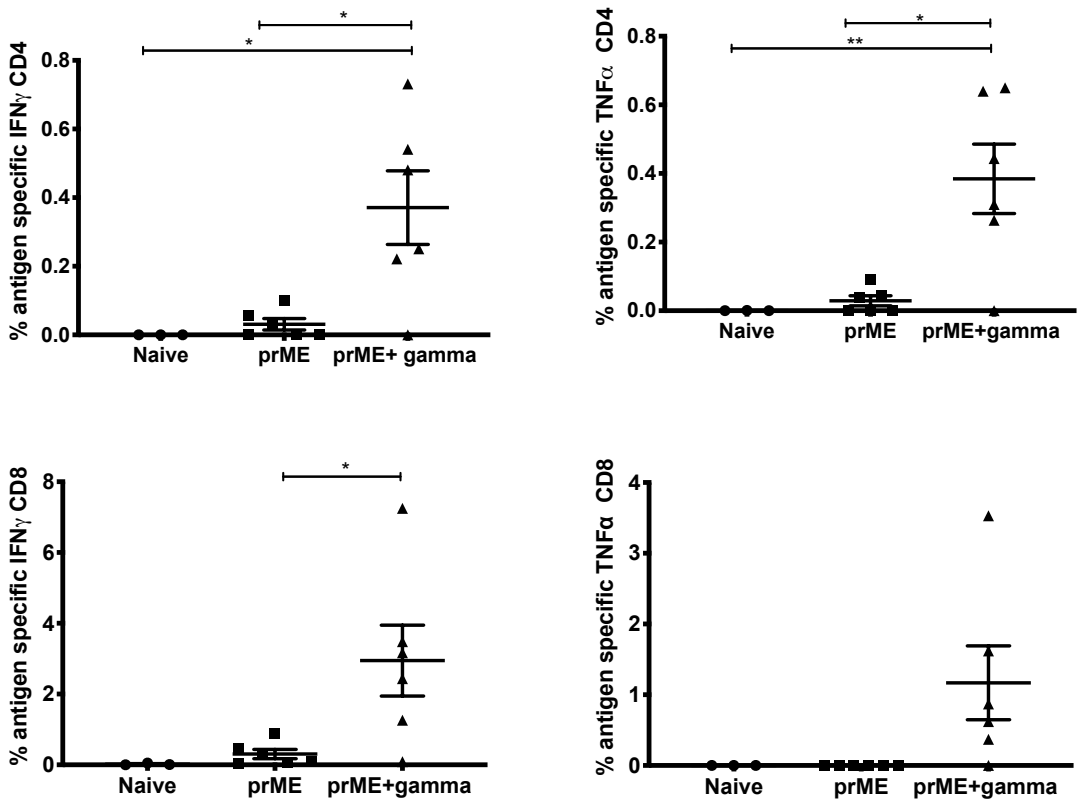


Figure 19. Opt-36γt enhances vaccine specific CD4$^+$ and CD8$^+$ T cell responses against prME

IFNAR -/- mice (n = 5-6 mice/group) were immunized once with either Zika prME vaccine alone (0.5 µg) or Zika prME (0.5 µg) and opt-36γt (11 µg). Antigen specific CD4$^+$ and CD8$^+$ T cell responses measured by intracellular cytokine staining 14 days after DNA injection and EP. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

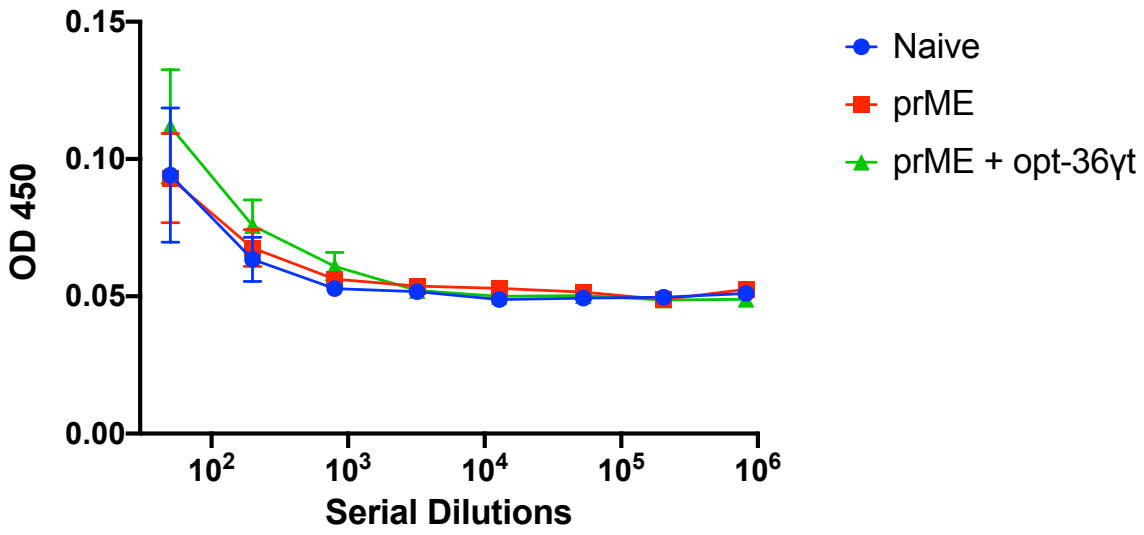


Figure 20. Single low dose of Zika prME vaccine does not induce antibody response

Antibody binding analysis of sera from IFNAR -/- mice (n = 5-6 mice/group) immunized once with either Zika prME vaccine alone (0.5 µg) or Zika prME (0.5 µg) and opt-36γt (11 µg). ELISA samples run in duplicate.

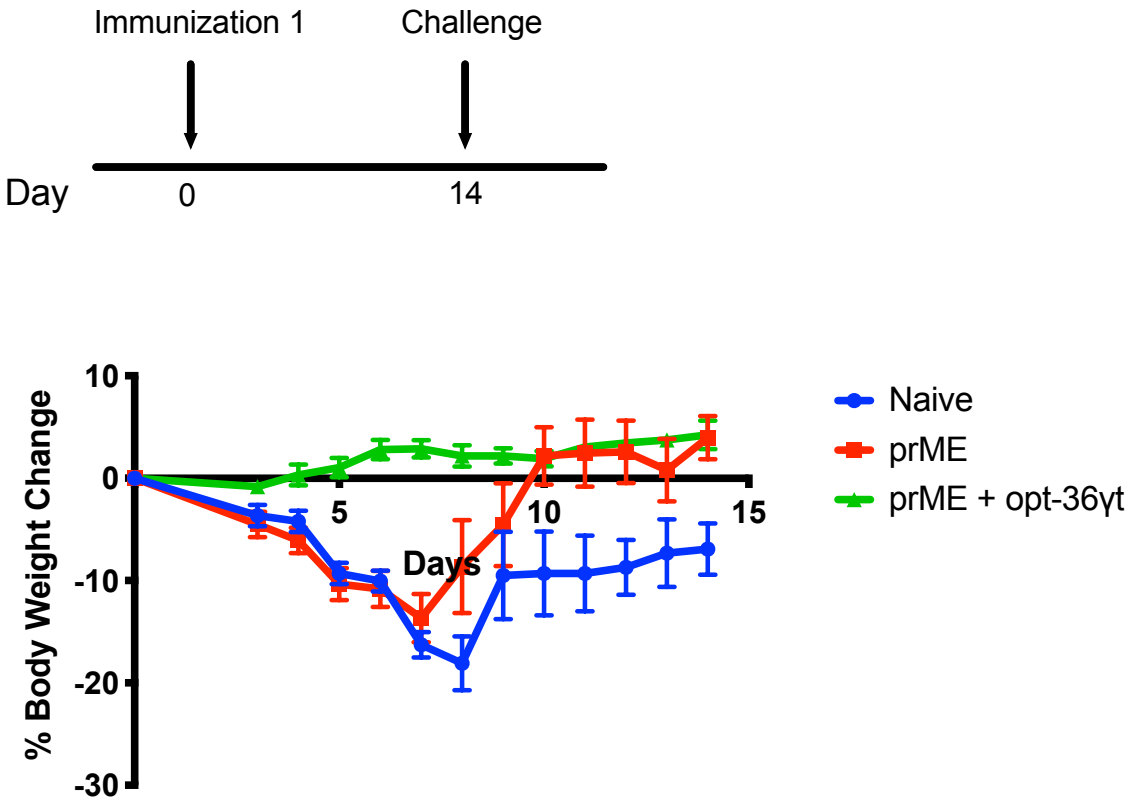


Figure 21. Opt-36γt adjuvanted mice maintain weight following Zika virus challenge

IFNAR-/- mice (n= 12-14 mice/group) were immunized once with either Zika prME vaccine alone (0.5 µg) or Zika prME (0.5 µg) and opt-36γt (11 µg) and challenged 14 days later with mouse adapted Zika virus strain, PR 209. Average body weight measurements of IFNAR -/- mice over the course of 14 days following challenge reported.

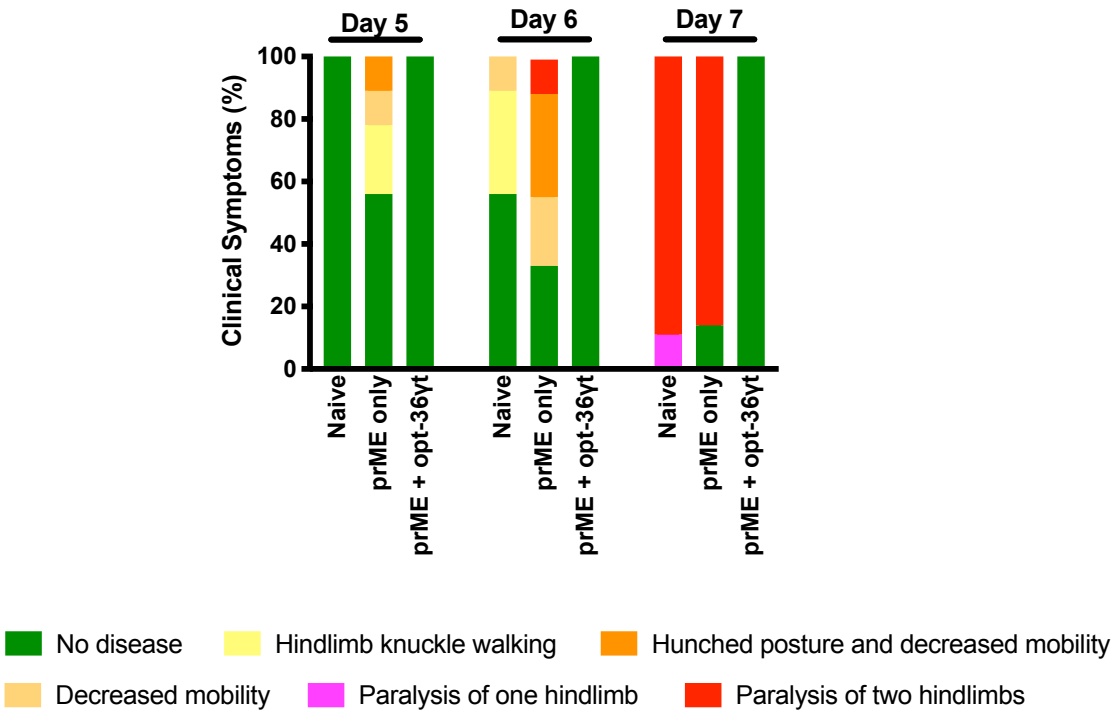


Figure 22. Opt-36γt enhances protection against clinical symptoms following Zika virus challenge

IFNAR-/- mice (n= 12-14 mice/group) were immunized once with either Zika prME vaccine alone (0.5 µg) or Zika prME (0.5 µg) and opt-36γt (11 µg) and challenged 14 days later with mouse adapted Zika virus strain, PR 209. Mice were monitored for 14 days following Zika virus challenge, and clinical symptoms due to challenge are depicted above for days 5-7.

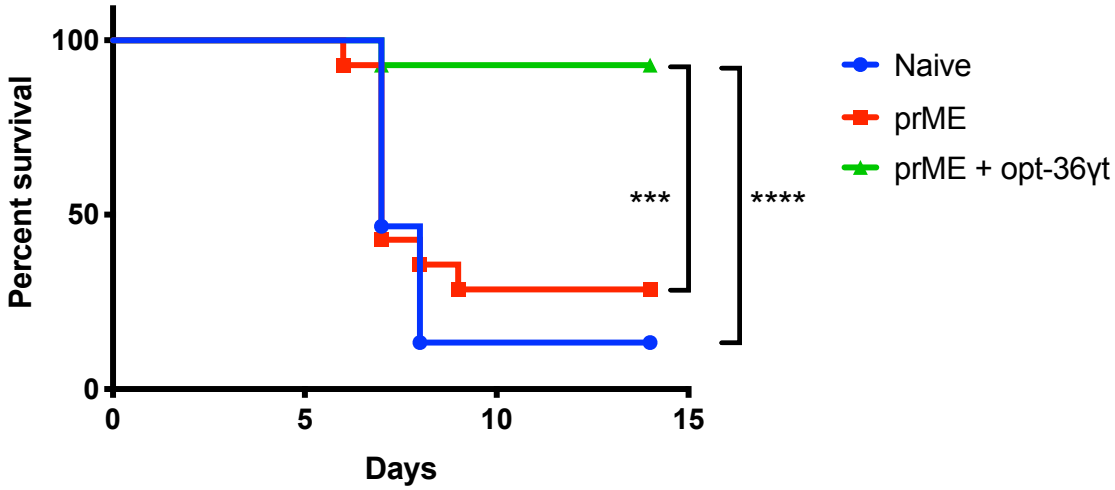


Figure 23. Codelivery of opt-36γt boosts overall survival following Zika virus challenge

IFNAR-/- mice (n= 12-14 mice/group) were immunized once with either Zika prME vaccine alone (0.5 µg) or Zika prME (0.5 µg) and opt-36γt (11 µg) and challenged 14 days later with mouse adapted Zika virus strain, PR 209. Mice were monitored for 14 days following Zika virus challenge for survival rates and were euthanized at predefined humane endpoints of either 20% loss of body weight or prolonged hind limb paralysis. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

“When I was a child, I spoke as a child, I understood as a child, I thought as a child; but when I became a man, I put away childish things.” 1 Corinthians 13:11, The Holy Bible

Scene: Lumena, age 28, realizing that she can get the updated Gardasil vaccine that protects against 9 strains of HPV, compared to the four strains in the original vaccine she took 5 years earlier at Student Health.

Student Health now administers Gardasil9 for students up to the age of 45? (Lumena reading Student Health’s website and realizing that she can get the updated Gardasil vaccine at no extra cost since the FDA approved the vaccine for a wider range of ages)

Oh my goodness, I gotta go set up my appointment now. I need all the protection I can get! Why have protection against 4 strains when you can have protection against 9? (Lumena furiously logging into the student health portal to set up an appointment to start the vaccine course)

Mommy, can you believe I can get this updated version of the vaccine, for FREE? I wish this was approved earlier so that I would have gotten the Gardasil 9 regimen from the start…(Lumena chatting with her mom about this vaccine)

Man, times change! I remember when I had to drag you to the doctor to scream through your shots, and now you are actively seeking them out! (Lumena’s mom still in amazement every time she willingly gets her vaccines.)

This life is hard, and I want to cover all the bases I can! (Lumena showing her mom the progess!)

CHAPTER 4- Intradermal synDNA vaccination generates Leishmania specific T cells in the skin and protection against Leishmania major

Introduction

Vaccination remains one of the greatest medical breakthroughs in human history, and has resulted in near eradication of many former lethal diseases in many countries including the complete eradication of smallpox. However, there remain a number of diseases for which there are no or only partially effective vaccines. There are numerous hurdles in vaccine development, of which knowing the appropriate immune response to target is one of them. Recently, tissue resident T cells have been shown to mediate high levels of protection for several infections, although the best ways to induce these cells is still unclear. Here we compare the ability to generate skin resident T cells in sites distant from the immunization site following intramuscular and intradermal injection using optimized SDNA vaccines. We observed that mice immunized intradermally with a synthetic consensus DNA HIV Envelope vaccine by electroporation (EP) are better able to maintain durable antigen specific cellular responses in the skin compared to mice immunized by the intramuscular route. We extended these studies by delivering a synDNA vaccine encoding *Leishmania* Glycosomal Phosphoenolpyruvate Carboxykinase (PEPCK) by EP, and again found that the intradermal route was superior to the intramuscular route for generating skin resident PEPCK specific T cells. When challenged with *Leishmania major (L. major)* parasites, we observed that mice immunized intradermally exhibited significant disease control, while mice immunized intramuscularly did not. The protection seen in intradermally vaccinated mice supports the viability of this platform to generate skin resident T cells, but and promote durable protective immune responses at relevant tissues sites.

The most successful approach to controlling infectious diseases is the development of protective vaccines, but unfortunately there remain several diseases for which no vaccines are available.

Resolving this deficit will require identifying the immune responses that provide protection, and then understanding how best to generate them. Recent studies with several diseases have reported that T cells residing in the tissues, resident memory T cells or T_{rm} cells (Gaide et al. 2015; Mackay et al. 2012; Jiang et al. 2012), can often provide greater protection than those that are circulating alone, although how to best generate T_{rm} cells through a vaccine remains poorly understood. For example, some studies suggest that intradermal immunizations or skin scarification may be particularly effective at generating skin T_{rm} cells, while other studies are less clear on this population's importance (Gray, Westerhof, and MacLeod 2018; Iborra et al. 2016; Takamura 2018). We wanted to address this issue in part by comparing the generation of skin resident T cells and protection against cutaneous Leishmaniasis following DNA immunizations via the intramuscular route, most often used for currently approved vaccines, and the intradermal route.

Leishmania infection occurs in over 88 countries, with an estimated 12 million people currently infected and over 350 million people at risk (Ponte-Sucre et al. 2017; Sundar and Singh 2014). The parasite is spread by the sand fly during the infective stage, metacyclic promastigotes, during a blood meal and is phagocytized by macrophages at the site of injection. The parasite then differentiates to the amastigote stage, multiplies, and infects other cells and tissues depending on the specific strain. The cycle is complete when a sandfly feeds on an infected host and picks up the amastigotes where they differentiate into promastigotes in the gut. Leishmania infection primarily impacts people in resource strained settings, and a number of barriers exist that bar access to the few treatments available for Leishmaniasis, including high costs, quality control issues, low production capacities, and physical geography (Sunyoto, Potet, and Boelaert 2018; Boer et al. 2011). However, evidence that many people who recover from clinical disease are generally protected from future reinfection suggests that a vaccine approach is feasible, and there are currently a large number of potential vaccines being tested in both experimental animal models and in clinical trials, though none are currently available for human Leishmaniasis (Bush

et al. 2017; González et al. 2010; López-Carvajal et al. 2018). Current thinking in the field suggests that a successful vaccine candidate for Leishmaniasis will need to generate $CD4^+$ IFN-γ, as IFN-γ activates macrophages' microbicidal activity and induces the production of nitric oxide (NO) and reactive oxygen species (ROS) that can destroy the parasite. Data from clinical trials that have used heat killed whole Leishmania parasite antigens have resulted in disappointing outcomes where any observed efficacy is short-lived, and as such, is unlikely to mount protective immune responses (Khalil et al. 2000; Bahar et al. 1996; Vélez et al. 2005). Live parasites have also been used in the past to induce immunity with success, however concerns with protracted and nonhealing lesions as well as parasite passage issues have caused this option to fall out of favor (Nadim et al. 1983; Khamesipour et al. 2005). A major problem in the field of vaccine development for Leishmaniasis has been the lack of an identified immunodominant Leishmania antigen. Recently, however, this deficit has been partially rectified with the discovery of a Leishmania protein, Leishmania Phosphoenolpyruvate Carboxykinase (PEPCK), which is an enzyme that is critical for gluconeogenesis. At the peak of Leishmania infection, nearly 20% of all Leishmania-reactive $CD4^+$ T cells are PEPCK specific in mice (Mou et al. 2015). Furthermore, the authors of this study found that peripheral blood mononuclear cells (PBMCs) from people that have recovered from zoonotic *L. major* infection recognize PEPCK and express higher IFN-γ and Granzyme B and increased cell proliferation compared to PBMCs from healthy non-infected people when stimulated with recombinant PEPCK protein, suggesting the potential of clinical benefit.

In order to determine if the route of vaccination, intramuscular (IM) or intradermal (ID), would influence the generation of skin resident T cells, we first tested the ability of a well-defined DNA vaccine developed for HIV to generate these cells by these two routes. We then created two synthetic consensus DNA plasmids that encode PEPCK, which represent sequences from six *Leishmania* parasites, including *L. infantum, L. donovani, L. major, L. mexicana, L. braziliensis*, and *L. panamensis*. These species represent both Old and New World *Leishmania* strains that

can cause cutaneous, mucosal, and visceral leishmaniasis. We immunized mice with these plasmids either intramuscularly or intradermally by CELLECTRA EP/SEP to evaluate vaccine-induced immunity and assess disease control following challenge. We found that mice immunized intradermally were better protected against *L. major* challenge compared to mice immunized intramuscularly. The levels of protection seen in intradermally immunized mice were similar to those seen in mice that were previously infected with parasites that exhibit superior control, as measured by lesion size and parasite burden, and suggest that the intradermal route may be more efficient at generating T_{rm} cells and protection against Leishmania induced disease compared to intramuscular vaccination.

Results

Intradermal vs intramuscular HIV Env vaccination at acute and memory time points. An important outcome for vaccination is to generate long-lived immunity to protect against future pathogen exposure. Therefore, we sought to examine in pilot experiments the immune responses elicited post vaccination as well as the response at a memory time point using defined constructs. C57BL/6 (B6) mice (n=3-5) were immunized with 25 μg of HIV Env DNA vaccine either intramuscularly with CELLECTRA electroporation (EP), or ID in the abdominal flank with surface electroporation (SEP), twice, two weeks apart (Figure 24). Spleens and skin at the injection site were collected ten days post final vaccination to analyze antigen specific responses. A quantitative ELISpot assay was performed to analyze the IFN-γ response in the spleen, and we found that mice immunized intramuscularly had an average of 1000 spot forming units (SFU) per million splenocytes, while intradermally vaccinated mice had an average of 250 SFU. In the skin, there was no significant difference between IM and ID immunized mice in the frequency of IFN-γ, IL-2, and TNF-α secreting $CD4^+$ T cells at the site of immunization, however there was a significant difference in the frequency of IL-2 and TNF-α producing $CD4^+$ T cells at the

contralateral site, suggesting some enhanced honing and mobility of these antigen specific T cells induced in the skin by ID immunization (Figure 25).

We next examined immune responses 75 days post-final vaccination to study the durability of the antigen specific responses (Figure 26). There was some contraction of the IFN-γ response, with an average of 800 SFU/million splenocytes for the IM group and 150 SFU/million for the ID group, but strikingly, there was a significant number of IFN-γ and TNF-α secreting $CD4^+$ T cells in the ID group compared to IM in the skin, suggesting that ID immunization has the potential to generate long lasting immunity at the site of vaccination in the skin. We did not observe a significant number of antigen specific $CD4^+$ T cells at the contralateral site at the memory time point in this model. Encouraged by these data, we next studied intradermal vaccination in a *Leishmania* model for which a mouse challenge exists.

Development of consensus *Leishmania* PEPCK vaccine. Challenges in *Leishmania* vaccine development are due in part to the lack of understanding of the antigens capable of eliciting potent Th1 IFN-γ $CD4^+$ T cell responses. However, recent work by Mou *et. al* has identified a conserved dominant protein, PEPCK, that elicited strong $CD4^+$ T cell responses. They found that ~17% of *Leishmania* reactive $CD4^+$ T cells were PEPCK specific at peak immune response during *L. major* infection. Given this robust response, we designed two constructs that encode consensus sequences for PEPCK to maximize coverage of both Old and New World strains of *Leishmania* (Tables 1 and 2). The PEPCK genes were inserted into a pVax1 backbone under the control of a CMV immediate-early promoter and IgE leader. Construct expression *in vitro* was confirmed using Western blot, to detect binding to PEPCK (Figure 27). Expression of PEPCK was observed in the lysates of transfected cells, but was not observed in the supernatant.

PEPCK plasmid delivery induces strong cellular IFN-γ responses following intramuscular vaccination. As IDM2 and PB differ by 7.5% in their amino acid sequences, we wanted to verify

that both plasmids were immunogenic, and to determine whether there might be an advantage to delivering both plasmids in a vaccine strategy. We performed intramuscular DNA delivery of either IDM2 or PB in C57BL/6 mice at a dose of 20 μg each, followed by EP, two times, two weeks apart. Ten days after final vaccination, we analyzed the degree of immune responses by isolating splenocytes. A quantitative ELISpot was performed to determine the number of PEPCK specific IFN-γ secreting T cells responded to vaccination (Figure 28). Both mice vaccinated with IDM2 or PB mounted a robust immune response, with an average of 2500 SFU/million and 3700 SFU/million splenocytes against IDM2 and PB respectively. Strikingly, there were a much greater number of T cells that responded to peptides in Pool 1 of PB, compared to the Pool 1 of IDM2, potentially suggesting additional epitopes that the T cells are recognizing. Based on this observation, we decided to combine the two plasmids into one vaccine for the reminder of the studies.

Delivery of both PEPCK plasmids as one vaccine induces strong systemic response following IM and ID vaccination. Given that the majority of vaccines administered today are delivered intramuscularly, we wanted to verify that our PEPCK constructs would be immunogenic whether administered IM or ID. We performed PEPCK (IDM2 and PB) vaccination either IM in C57BL/6 (B6) mice (n=5) with a dosage of 40 μg total of DNA (20 μg IDM2, 20 μg PB) or ID (n=5) with the same dosage, followed by IM-EP or ID-SEP, two times, two weeks apart. The ID-SEP device is less invasive and targets the epidermis. Ten days after final vaccination, we analyzed the degree of immune responses by isolating splenocytes. A quantitative ELISpot was performed to determine the number of PEPCK specific IFN-γ secreting T cells responded to vaccination (Figure 29). Both mice vaccinated IM or ID mounted a robust immune response, with an average of 1600 and 1800 SFU/million splenocytes against IDM2, and 2500 and 2200 SFU/million splenocytes against PB, respectively. T cell polyfunctionality has been shown to be protective in some models, thus we sought to assess the quality of T cells that were responsive to our

vaccination. We found that PEPCK specific $CD4^+$ T cells secreted IFN-γ, IL-2, and TNF-α (Figure 29). Furthermore, we observed a humoral response to PEPCK vaccination by IFA. Sera from PEPCK immunized mice were used to detect antibody binding to IDM2/PB transfected HEK293T cells. Natural infection with Leishmania induces a robust humoral response, although it is still unclear the role of antibodies in infection and protection. A large number of studies in mice have found that IL-4 mediated Th2 response, i.e. antibody skewing, to be detrimental to protection against Leishmania, however others have found antibodies to play a protective role in challenge models. These discrepancies may be attributed to the differences in immune responses generated by different parasite strains, and continue to be teased out. It is apparent however that a predominant Th1 $CD4^+$ IFN-γ response is protective in multiple Leishmania strains, and as such the primary focus and target of these studies.

Intradermal delivery of PEPCK elicits robust IFN-γ responses in the skin. With ~90% of all Leishmaniasis cases presenting in the skin, potential therapies for the infection should be able to induce immune responses in this important organ. The vaccine must also be able to induce T cells that are capable of homing to the point of parasitic infection, even when the vaccination site is not in close proximity. To address this, we compared the immune responses mounted in the skin at the site of immunization as well as contralateral sites in response to PEPCK vaccination given by either the IM or ID route. We found that ID immunization resulted in a high frequency of IFN-γ and TNF-α secreting PEPCK specific $CD4^+$ T cells in the skin at the immunization site (Figure 30). We did not detect any significant vaccine induced immune responses in the skin of mice immunized IM. More importantly, PEPCK specific $CD4^+$ T cells were found in the skin contralateral to the vaccination site, suggesting that this immunization strategy generated T cells able to home and travel throughout to non-inflamed skin.

Immune responses elicited by PEPCK vaccination are durable. As one of the major issues in Leishmania vaccine development is the durability of immune responses, we sought to examine

responses generated at a memory time point. We immunized mice as above and harvested spleens 45 days post final vaccination. As expected, IFN-γ responses contracted, with an average 1200 and 500 SFU/million splenocytes against IDM2 in the IM and ID groups, and 2000 and 800 SFU/million splenocytes against PB, respectively (Figure 31). In the spleen, both IM and ID groups were able to maintain IFN-γ, IL-2, and TNF-α responses, with the IM group maintaining the highest frequency. Splenic responses appear to be most affected in the ID group with a three fold contraction in IFN–γ responses measured by ELISpot, compared to the IM group that was able to maintain similar numbers of antigen specific IFN-γ secreting T cells. However, this was not true in the skin. Mice that were immunized ID were able to maintain a more significant frequency of IFN-γ producing $CD4^+$ T cells in comparison to mice immunized IM (Figure 32).

Intradermal vaccination of PEPCK DNA vaccine leads to better disease control compared to intramuscular vaccination. To determine the levels of protection against *L. major* challenge by each delivery route, naïve, vaccinated and previously challenged control mice were challenged in the ear. The challenged ear from each group was collected to analyze the parasite burden at the site of challenge. In response to *L. major* challenge and inflammation, the ear will form parasitic lesions, thicken, and become inflamed. Naïve mice were unable to control disease, and as such exhibited thickened ears, whereas ID immunized and immune mice maintained a much lower ear thickness, and IM immunized mice had an intermediate presentation (Figure 33). ID immunized mice were able to lower their parasite burden by nearly three logs, very similar to the parasite burden seen in immune mice, while IM immunized mice lowered their parasite burden by two logs. More strikingly, at a memory time point, ID immunization is still able to significantly reduce parasite burden, while IM immunized mice seem to lose parasite control, suggesting long lasting protection against challenge. These experiments suggest that ID immunization has the potential to generate long-lasting control of *L. major* infection, by a simple and less invasive immunization approach.

Discussion

Leishmaniasis is an important neglected tropical disease that can cause severe disfiguring lesions or fatal visceral infections, and although there have been substantial efforts for decades to develop a Leishmania vaccine, no options exist yet for this infection. There is evidence that a primary infection with Leishmania parasite leads to protection against future reinfection, suggesting the possibility of a vaccine; however there have been reported cases of people being infected with multiple parasite species, highlighting the need for a vaccine that can provide broad protection across species. As $CD4^+$ IFN-γ production is associated with protection and disease resolution, many candidates have been screened for their ability to elicit this immune response. A recent study found that $CD4^+$ T cells that resided in the skin following a primary infection in mice infected with *L. major* provided the best protection over disease burden (Glennie et al. 2015). Thus, after mice had resolved an infection, the authors of the study were able to find Leishmania-specific $CD4^+$ T cells present at skin sites distant from the site of the primary lesion. Furthermore, skin grafting experiments have demonstrated that tissue resident memory T cells (T_{rm} cells) were maintained in the absence of persistent parasites and could provide significant protection independent of any systemic immune responses. Taken together with multiple studies showing the superior protective capacity of tissue resident memory T cells against many other infections, these results indicate that a candidate Leishmania vaccine should elicit T_{rm} cells.

In these studies we provide insight on the potency and durability of intradermal vaccination using the SDNA platform in two different models. We observe that ID immunization is able to induce systemic immune responses post vaccination, as well as maintain immune responses in the skin at memory time points. We demonstrate that ID delivery of PEPCK DNA is able to protect mice from disease burden at levels similar to those seen in immune mice, and superior to IM injection, both soon after vaccination as well as at later time points. ID immunization resulted in reduced ear thickness post *L. major* challenge, and a three-log reduction in parasite burden in comparison

to naïve mice. In contrast, while IM immunization generated a strong systemic cellular response, the T cell responses in the skin were limited, and protection against parasite burden was not as robust as that seen following ID immunization. We have previously demonstrated that the most optimal protection against Leishmania challenge in mice is likely elicited from a combination of skin resident and circulating effector T cells (Glennie, Volk, and Scott 2017). With this work, we have shown that while IM and ID PEPCK delivery both induce similar levels of antigen specific cellular responses systemically, only ID immunized mice are able to significantly drive PEPCK specific T cells in the skin, resulting in enhanced protection against *L. major* challenge. These results indicate the superior ability of ID DNA vaccination in the *Leishmania* context to induce skin resident T cells and protective immune responses, and provide practical information needed for translating this vaccine from a mouse model to human study.

While our studies have identified an appropriate T cell population to target in a vaccine, the other major hurdle has been identifying appropriate vaccine candidates that generate effective long-term immunity. A substantial step forward was the identification of a Class II restricted immunodominant antigen (PEPCK) in Leishmania (Mou et al. 2015). At peak infection in mice, over 12% of the $CD4^+$ T cells in the blood recognized PEPCK, and vaccination with PEPCK protein could induce significant protection against a *L. major* challenge. Moreover, PEPCK is conserved in many different species of Leishmania, including those that cause both cutaneous and visceral disease, making it likely that it could be part of a pan-leishmania vaccine. Consensus vaccines have the potential to induce cross protection across species, making it possible to envision a single vaccine that could provide broad protection against a number of Leishmania parasite strains. Therefore, we chose a consensus PEPCK vaccine for our studies of how best to induce skin resident T cells. While intradermal SEP PEPCK vaccination was able to limit disease against *L. major* challenge and reduce parasite burden post vaccination and at a memory time point, mice that were previously infected with parasites still had lower parasite burden, suggesting room for improvement. As previously challenged mice are whole parasite experienced, including

other antigens besides PEPCK may help improve vaccine-induced immune responses and further reduce parasite burden as well as lesion size and inflammation. As the mice are challenged with two million parasites, it may also be the case that the immune system “sees” much more antigen overall in previously challenged mice compared to the amount delivered in our vaccination studies. Dosing studies that examine the impact of delivering higher doses of total antigen are warranted. Although the ratio of responses to the different peptide pools remain the same, the level of cellular responses observed when IDM2 and PB are delivered separately are higher than when the two are combined, suggesting that there may be some antigen interference occurring in the one injection site schema. Future studies that investigate whether delivery of the two plasmids at separate sites can enhance immunogenicity and lead to better disease control should be explored.

The notion that T cells primed in a specific site are more likely to return to that site as effector cells is well established, and therefore it is consistent that the ID route may be superior to the IM route for generating skin resident T cells. An excellent example of this concept comes from the earliest vaccine, the use of scarification with vaccinia, which not only generates resident memory T cells at the site of immunization, but also leads to the accumulation of Trm cells in sites distant from the immunized skin (Jiang et al. 2012). Similarly, our studies demonstrate that ID immunization also generates global immunity in the skin, in a platform that could easily be transferred to human vaccination. Aside from a better ability to generate skin resident T cells, there may be additional advantages to the ID route. As the skin contains more antigen-presenting cells compared to the muscle, it is believed that ID delivery may result in a more efficient vaccination, potentially leading to a dose-sparing effect. This could have a significant impact in the pandemic vaccine field, where there is always a fear of the limited supply of available doses. Data from rabies and influenza clinical trials suggest that ID delivery can maintain similar antibody titers while reducing dose, when compared to IM delivery (Resik et al. 2010). Intradermal vaccination is also considered more tolerable than intramuscular, which can have a positive

impact on vaccination rates and adherence, and with the continued development of intradermal devices that reduce the need for needles, reduced needle stick injuries and greater safety may follow.

As the climate continues to change, *Leishmania*, endemic to tropical and subtropical regions, will continue to spread throughout the globe. More therapies are desperately needed to treat Leishmaniasis patients, and an effective vaccine could dramatically reduce the burden associated with this disease. Our studies in mice provide a foundation for how best to translate this experimental vaccine into a practical effective vaccine for human Leishmaniasis, and demonstrate the superior nature of the ID route for generating skin resident T_{rm} cells.

*Modified from: Louis L, Clark M, Wise MC, Glennie N, Wong A, Broderick K, Uzonna J, Weiner DB, Scott P. Intradermal synDNA vaccination generates skin-resident T cells and protection against *Leishmania major*. *Infection and Immunity*. 2019; 87(8) 1-14

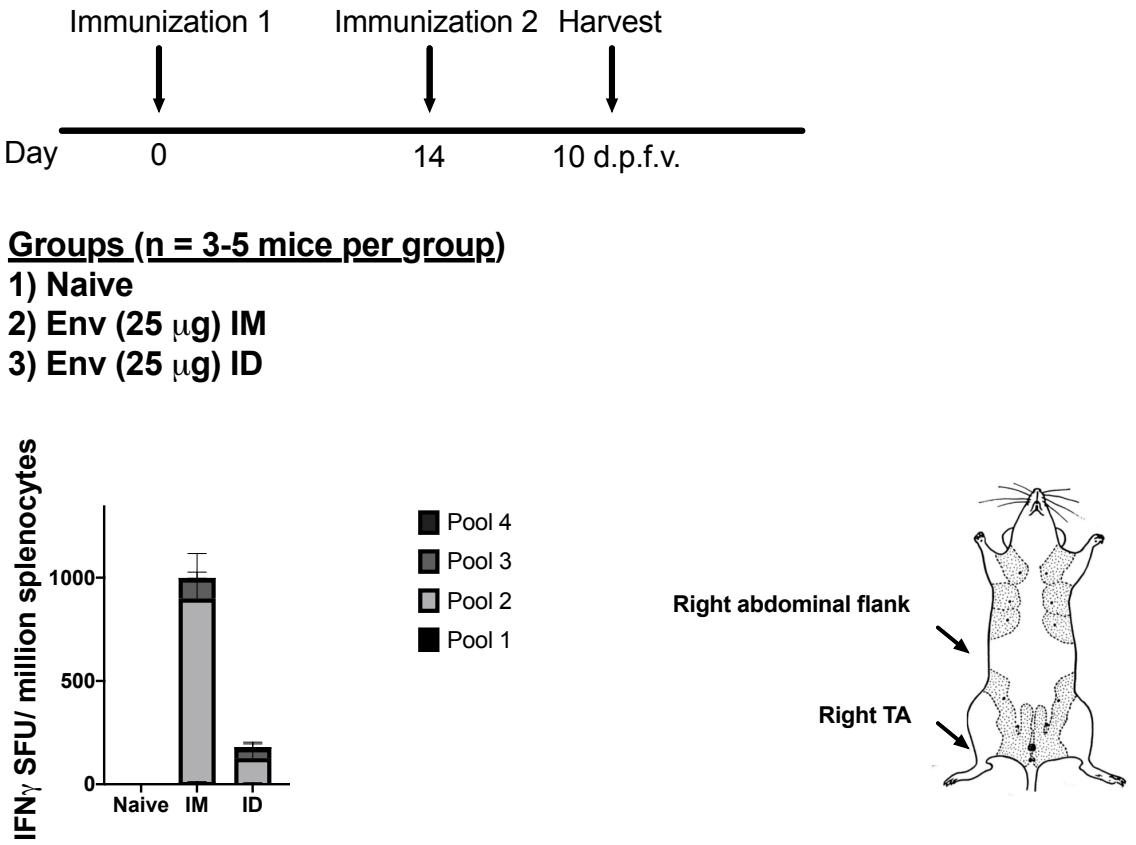


Figure 24. Intramuscular and intradermal delivery of Env DNA vaccine induces IFN-γ responses in the spleen

C57BL/6 mice (n = 3-5 mice/group) received an intramuscular or intradermal DNA injection and EP/SEP of 25 μg HIV Env vaccine alone two times at two week intervals in the tibialis anterior muscle or abdominal flank. Spleen and skin were harvested 10 days after the final immunization. ELISpot analysis of IFN-γ T cell responses against pooled Env peptides shown above.

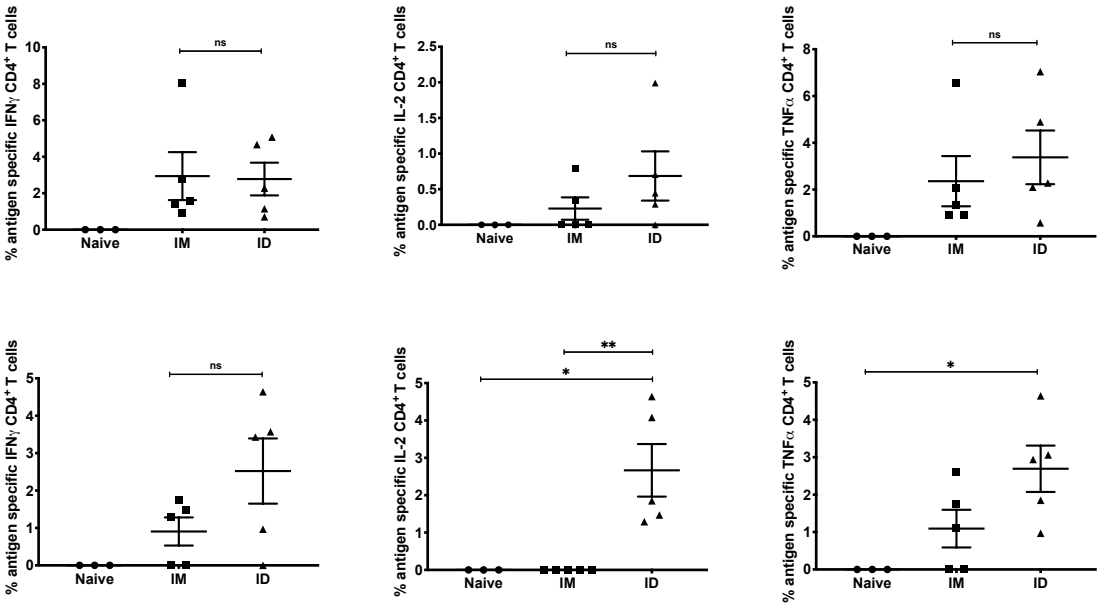


Figure 25. Intradermal delivery of Env DNA vaccine induces antigen specific responses in the skin at immunization and contralateral sites.

C57BL/6 mice (n = 3-5 mice/group) received an intramuscular or intradermal DNA injection and EP/SEP of 25 μg HIV Env vaccine alone two times at two week intervals in the tibialis anterior muscle or abdominal flank. Skin was harvested 10 days after the final immunization. Antigen specific $CD4^+$ T cell responses measured by intracellular cytokine staining 10 days after final immunization. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

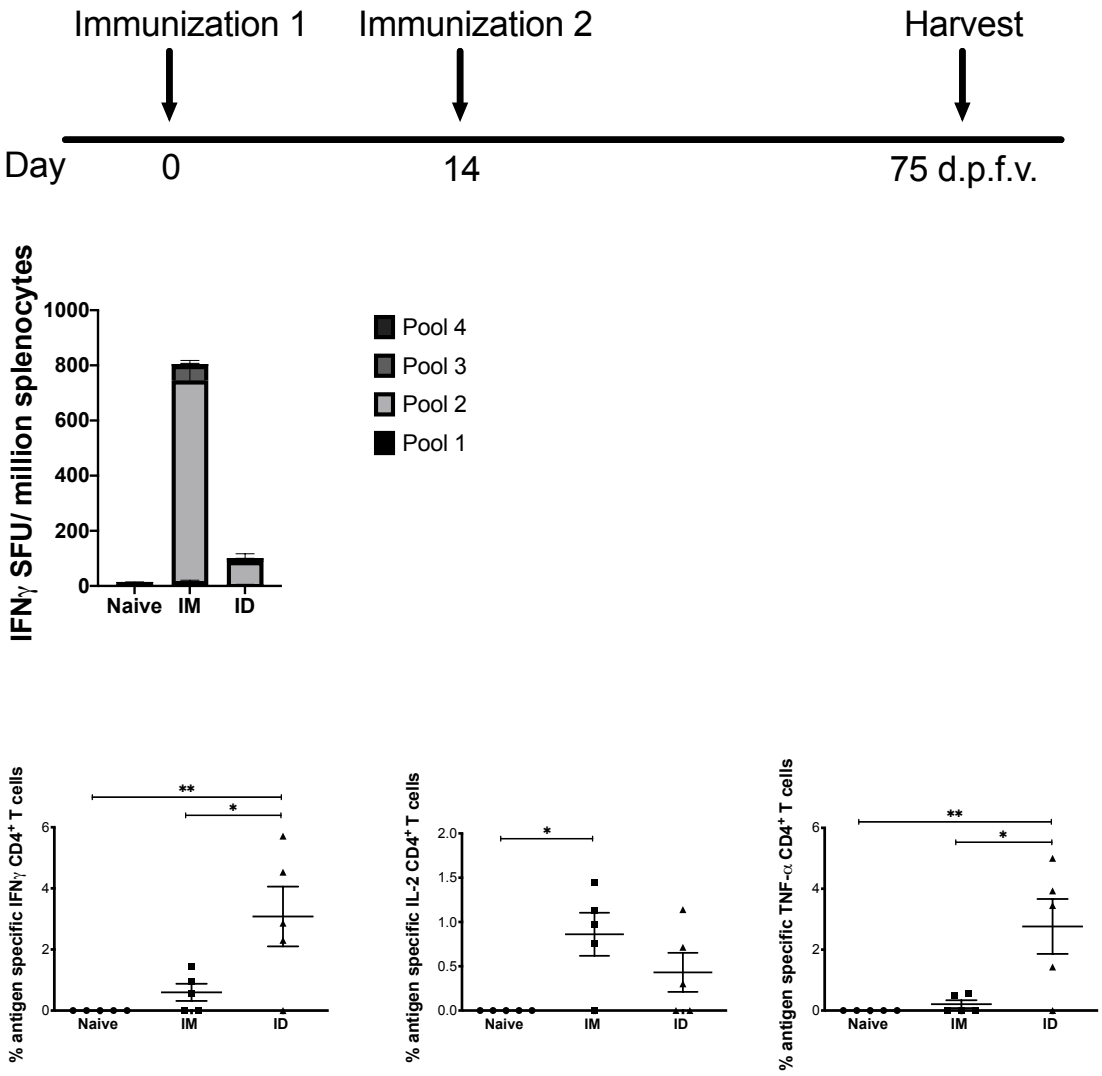


Figure 26. Intradermal delivery of Env DNA vaccine induces antigen specific responses in the skin at immunization site at a memory timepoint.

C57BL/6 mice (n = 5 mice/group) received an intramuscular or intradermal DNA injection and EP/SEP of 25 µg HIV Env vaccine alone two times at two week intervals in the tibialis anterior muscle or abdominal flank. Spleen and skin was harvested 75 days after the final immunization. ELISpot analysis of IFN-γ responses in the spleen. Antigen specific $CD4^+$ T cell responses measured by intracellular cytokine staining 75 days after final immunization. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

Parasite	Leishmaniasis manifestation
L. infantum	Visceral, cutaneous
L. donovani	Visceral
L. major	Cutaneous
L. mexicana	Cutaneous
L. panamensis	Cutaneous
L. braziliensis	Mucosal

Table 2. Common forms of Leishmaniasis manifestation

Different strains of Leishmania parasites can drive different Leishmaniasis presentation. These strains were chosen for the consensus vaccines to cover a broad range of clinical presentations.

infantum	donovani	major	IDM2	mexicana	braziliensis	PB	panamensis
donovani	0.008						
major	0.019	0.027					
IDM2	0.025	0.025	0.017				
mexicana	0.061	0.061	0.051	0.035			
braziliensis	0.092	0.090	0.086	0.075	0.098		
PB	0.094	0.092	0.086	0.075	0.098	0.006	
panamensis	0.084	0.086	0.075	0.065	0.088	0.015	0.010

Table 3. Diversity Table of amino acid differences between Leishmania strain PEPCK and consensus vaccines

The evolutionary history was inferred by the neighbor joining method and all evolutionary distances were computed using the Poisson correction method and are in the units of amino acid substitutions per site. All positions with less than 30% site coverage were eliminated.

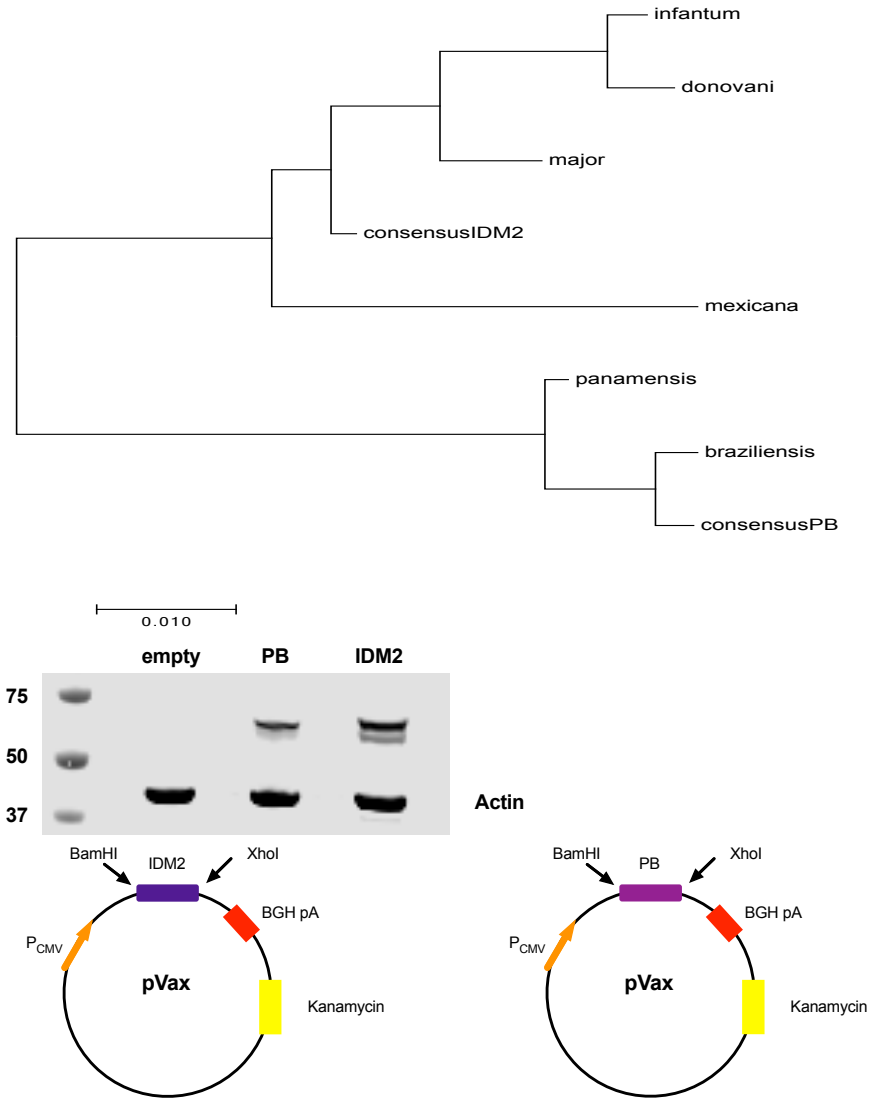


Figure 27. Optimized PEPCK constructs express *in vitro*

Diversity among Leishmania parasite and consensus sequences. The evolutionary history was inferred by the neighbor joining method and all evollutionary distances were computed using the Poisson correction method and are in the units of amino acid substitutions per site. All positions with less than 30% site coverage were eliminated. Map of plasmid construct design for consensus sequences. Each plasmid contains a CMV promoter followed by an IgE leader sequence beside the consensus PEPCK sequence. HEK293T cells were transfected with either IDM2 or PB consensus plasmids that contained a C-terminal HA tag for detection. Lysates from these cells were used in Western blot for detection of plasmid expression under reducing conditions.

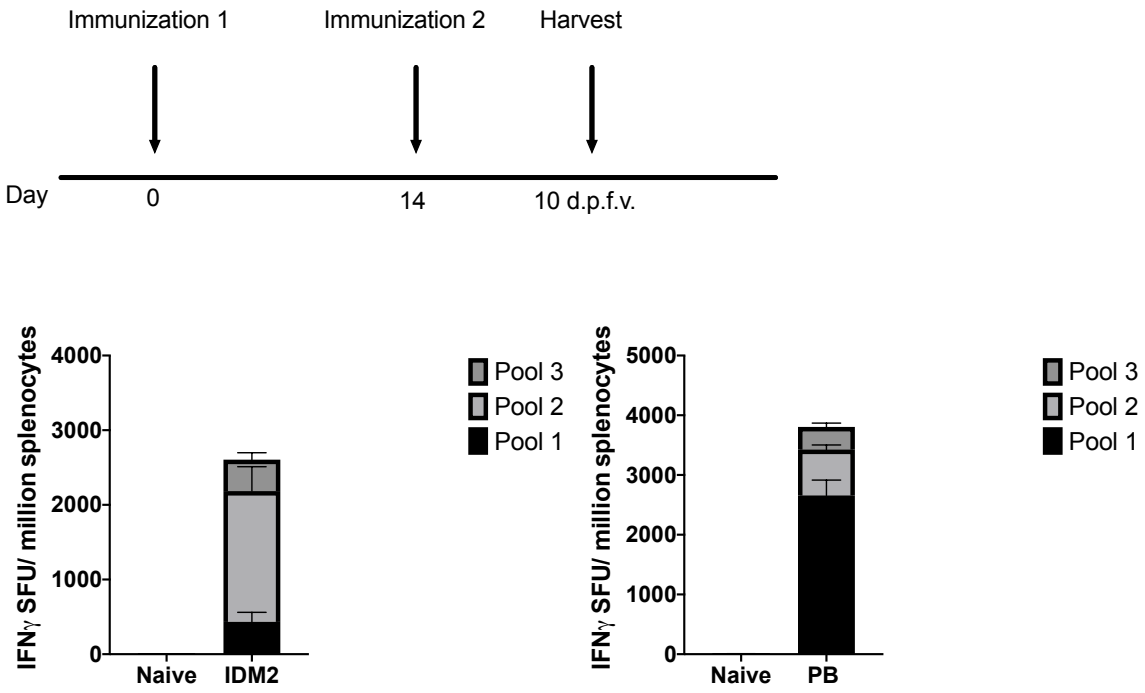


Figure 28. IDM2 and PB consensus PEPCK vaccines are immunogenic and induce IFN-γ responses in the spleen

C57BL/6 mice (n = 5 mice/group) received an intramuscular DNA injection and EP of either 20 µg of IDM2 vaccine (consensus of PEPCK from L. infantum, donovani, major, and mexicana) or 20 µg of PB vaccine (consensus of PEPCK from L. panamensis and braziliensis) two times at two week intervals in the tibialis anterior muscle. ELISpot analysis of IFN-γ responses 10 days after final immunization.

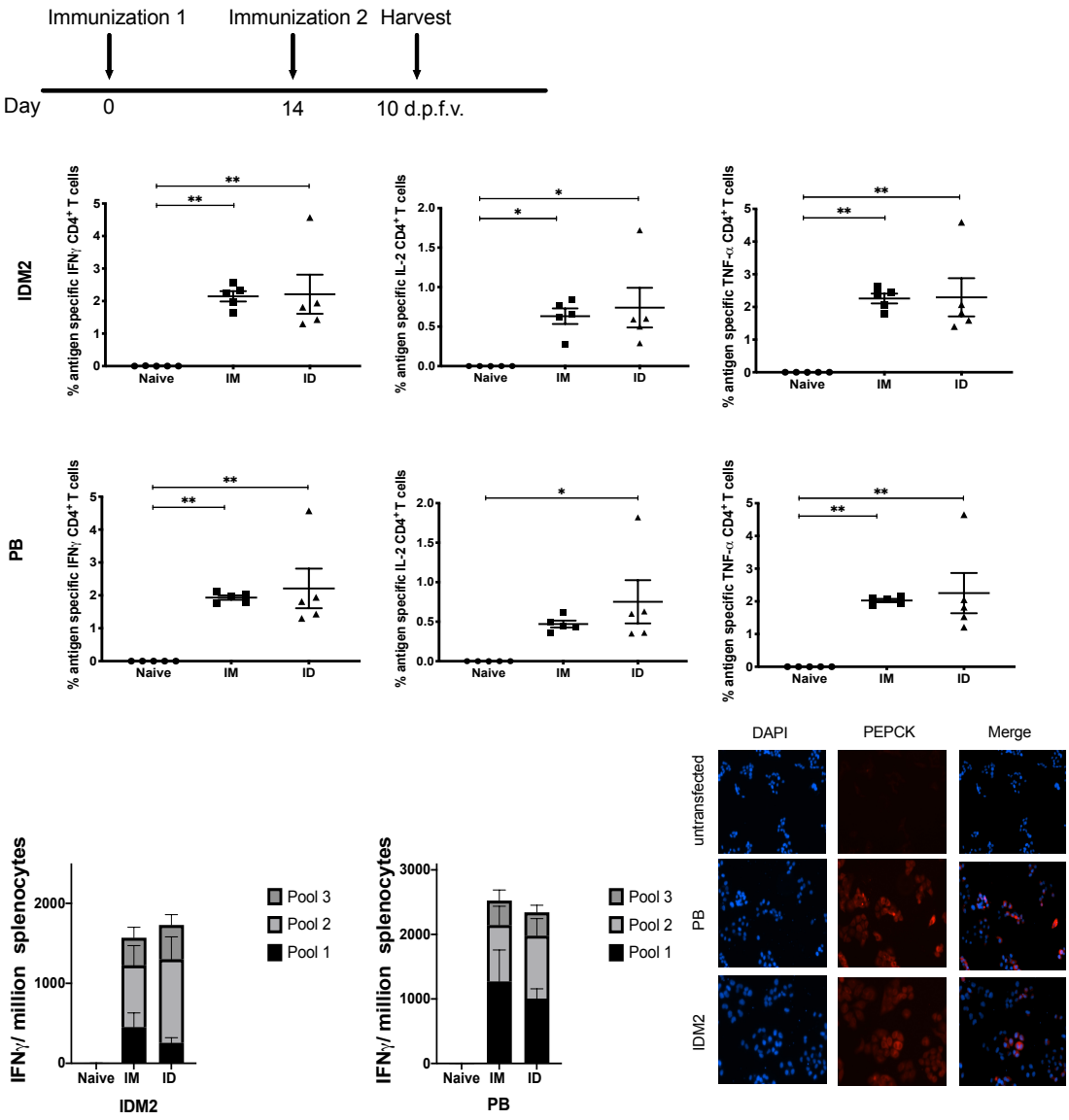


Figure 29. Intramuscular and intradermal PEPCK immunization induces systemic antigen specific responses

C57/BL6 mice (n = 4-5 mice/group) were immunized twice two weeks apart either intramuscularly or intradermally. Spleens and sera were harvested 10 days post final vaccination to analyze antigen specific T cell responses. The frequency of PEPCK specific IFN-γ responses after vaccination was determined by ELISpot assay in response to pooled PEPCK peptides. PEPCK specific $CD4^+$ T cell responses to each consensus plasmid by intracellular cytokine staining after peptide stimulation. HEK293T cells were transfected with PEPCK plasmids and sera from mice immunized with the constructs was used to detect antibody binding to PEPCK by IFA.

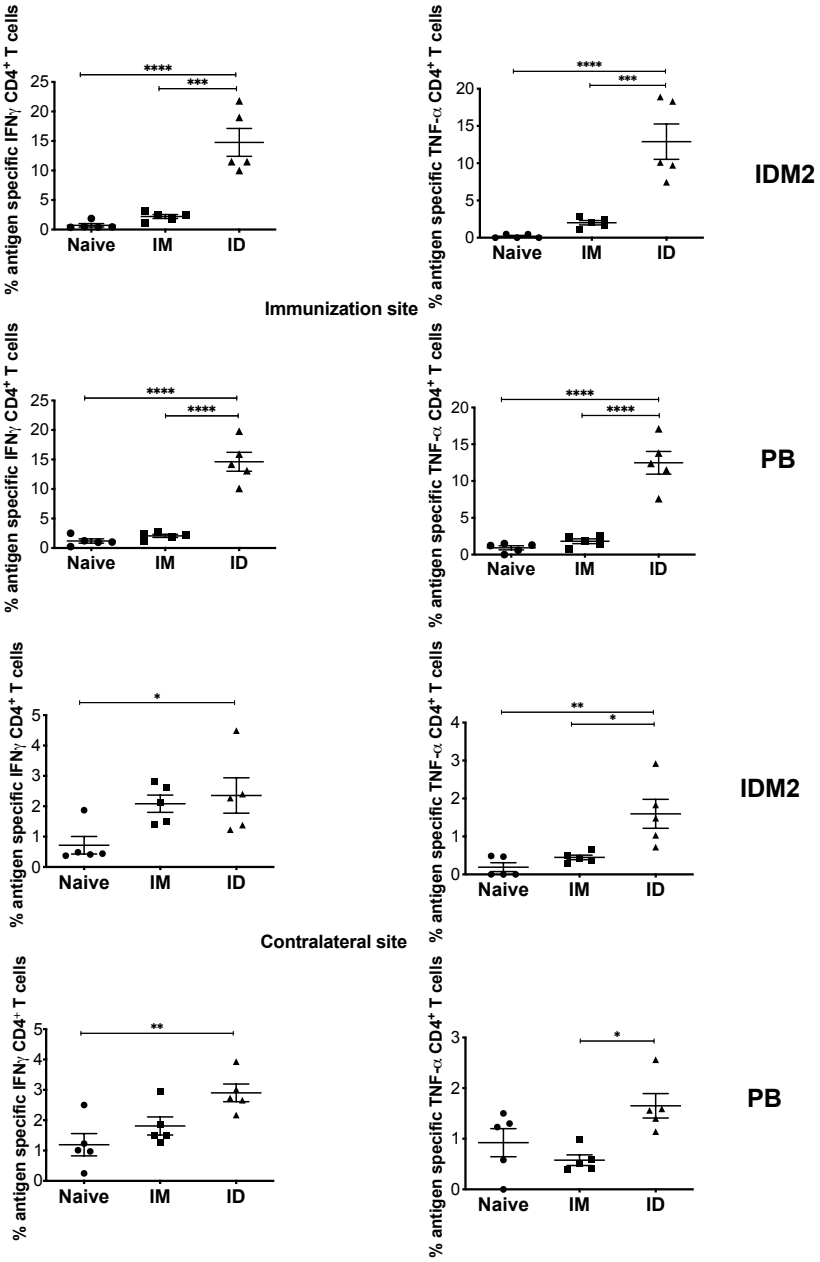


Figure 30. Intradermal PEPCK vaccination induces antigen specific CD4+ T cells at immunization and contralateral sites in the skin

C57/BL6 mice (n = 4-5 mice/group) were immunized twice at a two week interval either IM or ID EP. Flank or tibialis anterior (TA) skin from vaccination and contralateral site was harvested 10 days post final vaccination and analyzed for PEPCK specific $CD4^+$ T cell responses by intracellular staining post PEPCK peptide stimulation.

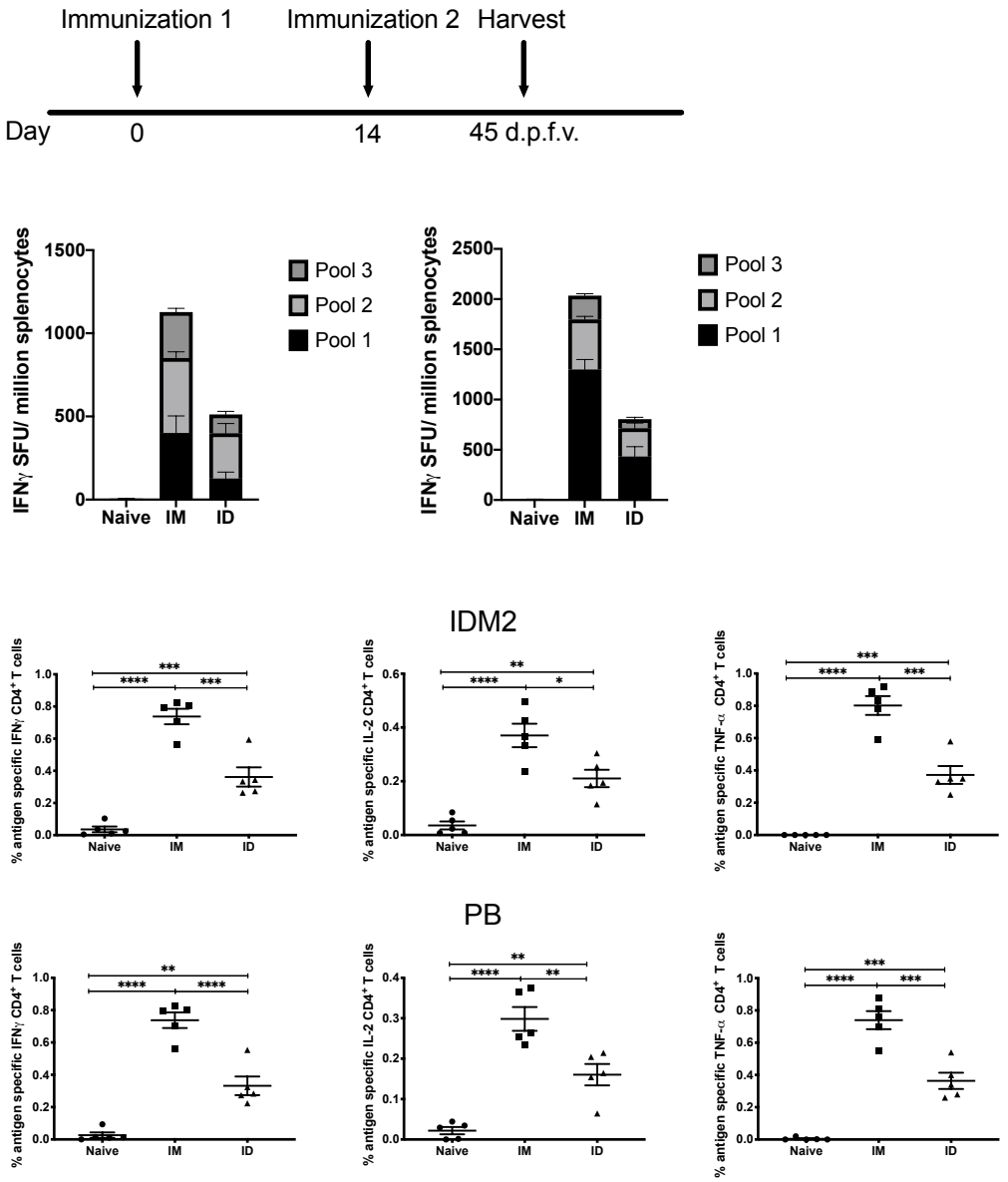


Figure 31. PEPCK specific IFN-γ responses are maintained in the spleen at a memory timepoint

C57/BL6 mice (n = 4-5 mice/group) were immunized twice two weeks apart either intramuscularly or intradermally. Spleens were harvested 45 days post final vaccination to analyze antigen specific IFN-γ T cell responses in memory phase. The frequency of PEPCK specific IFN-γ responses induced after vaccination was determined by ELISpot assay in response to pooled PEPCK peptides. PEPCK specific $CD4^+$ T cell responses to each consensus plasmid by intracellular cytokine staining after peptide stimulation are shown above. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

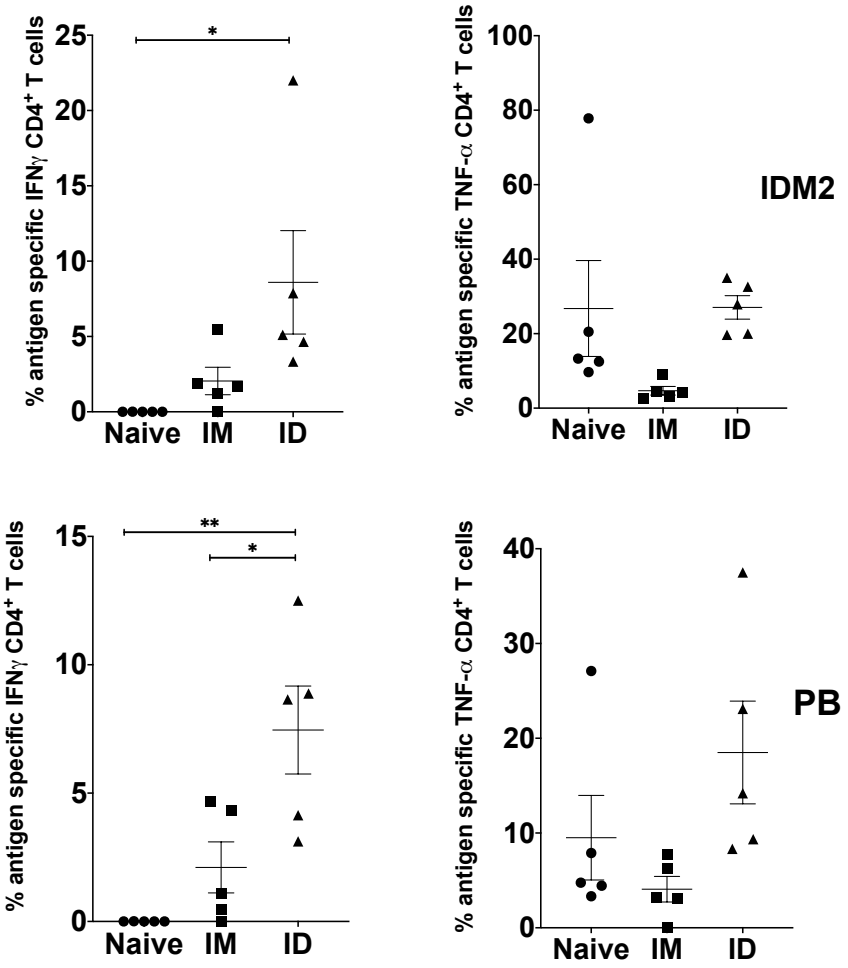


Figure 32. PEPCK specific IFN-γ responses are maintained in the skin at a memory timepoint

C57/BL6 mice (n = 4-5 mice/group) were immunized twice two weeks apart either intramuscularly or intradermally. Skin at the immunization site was harvested 45 days post final vaccination to analyze antigen specific IFN-γ T cell responses in memory phase. PEPCK specific $CD4^+$ T cell responses to each consensus plasmid by intracellular cytokine staining after peptide stimulation are shown above. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

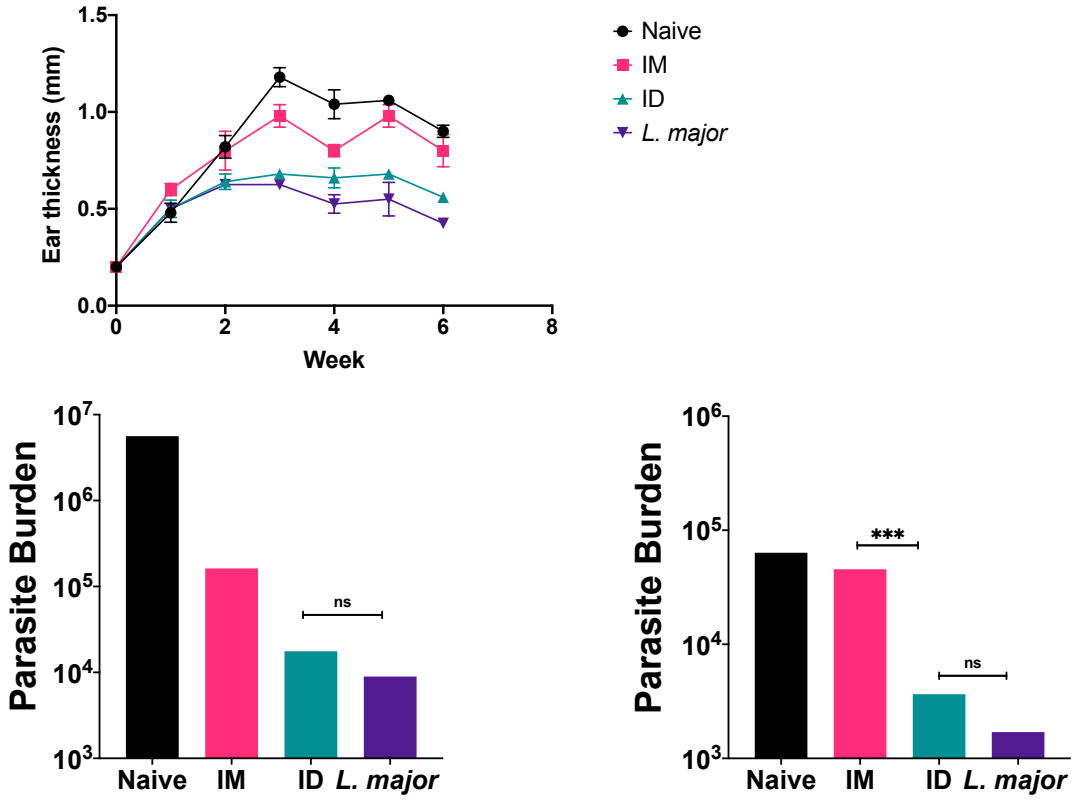


Figure 33. Intradermal PEPCK vaccination protects mice against *L. major* challenge

C57/BL6 mice (n =5 mice/group) were immunized once intradermally or intramuscularly with PEPCK and challenged with 2 million L. major parasites either 10 days or 45 days post immunization. Ear thickness curve plotting ear thickness over the span of challenge. The parasite burden in the challenged ear was quantified on day 55 post final immunization or day 90 following final immunization. Error bars represent standard error of mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$

CHAPTER 5 – Discussion and Future Directions

The potential of the DNA platform to revolutionize the way that vaccines are made and delivered has not been fully realized today as early setbacks in scaling DNA from small preclinical animal models to large animals and humans relegated DNA to a background role as boost immunizations to viral vector primes in vaccine schemes. However, advancements in the technology, including electroporation, concentrated DNA formulations, and sequence optimization have significantly improved the outcomes of DNA vaccination to levels seen in viral vector vaccine delivery. DNA vaccines maintain a stellar safety profile, as they are nonlive and nonreplicating, eliminating the risk of attenuation reversion in high-risk populations that may be immunocompromised. Over 30,000 people have been vaccinated with DNA with no serious adverse effects, alleviating worries of DNA integration that has long concerned the field. DNA vectors are not immunogenic, allowing for repeated administration without immune interference or concerns about previous viral exposure. Additionally, DNA is very stable, limiting the need for a cold chain, which in turn makes it an ideal candidate for delivery in resource strained settings.

As the quest for novel adjuvants that enhance vaccine induced responses and skew the response in the direction that favors protection continue, investigation of novel cytokines that can elicit these responses will grow. In this thesis, I've highlighted the potential of the truncated IL-36 family (subject of chapter 3) to boost immune responses following intramuscular delivery of three separate SDNA vaccines, and show that truncated IL-36 gamma can enhance protection of a non protective dose of Zika SDNA vaccine against a lethal Zika challenge. We first demonstrate that truncation of IL-36 beta results in enhanced immune responses against a HIV DNA vaccine. We then show that truncated IL-36 beta and IL-36 gamma exert their influences on $CD4^+$ and $CD8^+$ T cells respectively to enhance cellular responses. In an influenza DNA vaccine model, truncated IL-36 gamma was able to increase antibody binding titers, while maintaining the avidity of the

antibodies, whereas codelivery of truncated IL-36 alpha and IL-36 beta were detrimental to antibody avidity. Lastly, we show that a non-protective dose alone of a Zika DNA vaccine does not generate significant cellular responses, nor protect mice against a Zika challenge. The combination of Zika vaccine with truncated IL-36 gamma was able to synergize to mount a robust cellular response that was able to protect mice against challenge.

Future IL-36 applications

As the IL-36 cytokine family is primarily expressed in the skin, it is highly conceivable that infections and diseases that originate in the skin may be great targets to further study IL-36 biology and investigate adjuvant activity. Merkel cell carcinoma, driven in part by the Merkel cell polyomavirus in most cases, is a relatively rare form of cancer that tends to occur on the face, head, and/or neck. While the virus that causes this cancer is very common, it is not completely understood how or why it can drive tumorigenesis in certain adults although increased sunlight exposure and older age are known risk factors (Becker et al. 2017). The cancer arises in Merkel cells, which are found in the epidermis of the skin. Given the importance of $CD8^+$ T cells in controlling tumor burden, a potential prophylactic or therapeutic vaccine for this cancer would likely need to elicit this T cell response. The data generated in my thesis project suggests that opt-36γt can elicit vaccine specific $CD8^+$ T cell response, and as such may play a role in future vaccine design studies in this model. The melanoma data from Wang et al showing that tumor burden is reduced when cancer cells expressing IL-36 gamma are transplanted lends further credence to the idea that IL-36 may impact cancer intervention responses.

The IL-36 family members have been described as potential immune sentinels that alert the immune system of danger. Such a role could be beneficial in vaccines where inclusion of an adjuvant such as IL-36 gamma could potentially jumpstart the immune response and drive either accelerated vaccine induced immunity or magnify overall response. Preliminary data that I've generated in a TERT (nonviral cancer antigen) SDNA vaccine model suggests that opt-36βt can

boost IFN-γ cellular responses compared to vaccine alone (Figure 34). Inclusion of this adjuvant and the other IL-36 members in a tumor challenge study in the context of SDNA would give exciting information to inform whether these cytokines can impact cancer outcome. A follow up to the Wang melanoma study (X. Wang et al. 2015a) where IL-36 gamma is administered as an adjuvant to a vaccine targeting melanoma antigens could tease out the ability of the cytokine to either prevent “tumor take,” slow down tumor growth, promote enhanced TILs, or even drive tumor clearance.

We observed enhanced antibody avidity in our influenza studies in mice adjuvanted with opt-36γt, suggesting a maturation of the antibodies induced. This leads to the question if mice immunized with opt-36γt experience greater somatic hypermutations in their antibodies, and if so, whether this is due to enhanced T follicular helper responses. Studies to examine the impact of IL-36 gamma on T follicular helper and B cells in the germinal center would help shed further light on the roles of IL-36 in enhancing immune responses during vaccination and under normal physiologic conditions.

Aberrant overexpression of IL-36 cytokines has been implicated in disorders of the skin, however we have seen great promise of opt-36γt and opt-36βt to enhance vaccine induced immune responses. Studies to examine whether IL-36 plasmid codelivery in the skin would mediate toxicity are very important. As many infections are mediated in the skin, adjuvant delivery in the skin could potentially further boost protection by recruiting CTLs and enhancing antibody production in the skin where the breach may occur.

Future directions: gene encoded adjuvants

As the number of infectious diseases and rates of certain cancers increase, the need for novel adjuvants that increase vaccine induced immune response, provide dose sparing benefits, and broaden immune response breadth in traditionally immunocompromised patients, will increase. The success of ASO1 as an adjuvant to the herpes zoster vaccine helps demonstrates some of the potential that adjuvants have to offer. Gene encoded adjuvants stand to offer even greater benefit as transfected cells with both vaccine target and adjuvant can sway the immune response at the same time, potentially leading to greater immune responses. Investigations of ligands that target TLRs appear particularly promising as adjuvants. Recent studies have shown that flagellin, a TLR5 agonist, can act as a gene adjuvant in a vaccine against *Toxoplasma gondii*, and enhance humoral and cellular responses (Maraghi et al. 2019). Yet another study investigated flagellin's ability to enhance vaccine responses as an adjuvant scaffold and observed augmented responses to an HIV epitope (Ajamian et al. 2018). Studies that investigate the use of novel cytokines to further current understanding of their roles in immunity as well as biological roles in homeostasis will continue to advance the field.

While more studies, both in appropriate preclinical models and clinical trials, are beginning to highlight the efficacy of SDNA vaccines in a number of infectious diseases and cancer, there is still much more work to be done to further refine the technology. When I first began my thesis work, there were relatively few studies that examined the immune response generated post SDNA vaccination in the skin. Of the studies that did investigate SDNA vaccination in the skin, primarily humoral responses were observed and reported. The lack of information regarding the cellular responses elicited by DNA vaccination in the skin led me to investigate the immune response generated post intradermal delivery of two DNA vaccines. Using an established HIV Env and a novel Leishmania PEPCK vaccine (the subject of chapter 4), we outline the development of an effective intradermal vaccine strategy that generates durable antigen specific

T cells in the skin, and enhances disease control in a Leishmania infection model. During my thesis work, results from a number of clinical studies were released that further reinforced the advantages of intradermal vaccine delivery (Figure A1).

Infection with Zika virus is generally a self-limiting infection characterized by fever, arthritis, and rash. However, there have been many reported cases of Zika virus induced Guillian-Barre Syndrome as well as congenital birth defects during pregnancy. There are currently no approved vaccines or therapeutics for this infection, and so a lot of work has been done to rectify the situation. A clinical study that examined immune responses to an intradermal vaccine delivery of a Zika SDNA vaccine in healthy volunteers found that 100% of the participants developed binding antibodies against the vaccine, and 62% of them developed neutralizing antibodies following vaccination. These antibodies were able to protect IFNAR -/- mice against Zika challenge.

The 2014 Ebola outbreak quickly spurned multiple groups and agencies to develop vaccine candidates and monoclonal antibody (mAb) therapies that could induce protective immune responses against the virus. There has been great encouraging data suggesting that these interventions, both on the vaccine and mAb arm, are effective, however most of the vaccine studies have assessed immune responses generated post intramuscular immunization. A clinical study published earlier this year assessed the immune responses generated after intramuscular and intradermal vaccination of an Ebola glycoprotein SDNA vaccine. The authors found that while intramuscular and intradermal vaccination both induced antibody responses, the volunteers in the ID cohort had the fastest and steepest rise in antibody production compared to IM. This rapid increase in antibodies will likely prove critical in the context of active outbreak.

The study presented in this thesis is the first to show that intradermal delivery of Leishmania PEPCK can induce PEPCK specific $CD4^+$ T cells in the skin. After two immunizations of two separate consensus DNA plasmids in the muscle, we observed the induction of robust systemic cellular responses that highlighted immunogenic epitopes in areas of the protein not previously

described. Recognizing the need for a simpler vaccine strategy, we combined the two plasmids into one injection, and observed the maintenance of cellular responses to respective peptide pools. Importantly, although intradermal and intramuscular PEPCK vaccination generated similar responses systemically, only mice immunized in the skin were able to elicit these responses in the skin, which is critical for infections that originate in the skin. We also determined that the responses generated by intradermal delivery are durable and are maintained in the skin at a memory time point, which is crucial in the Leishmania field given the number of candidates that show initial protection that wanes over time. Following a Leishmania parasite challenge, mice that were immunized intradermally maintained better disease control compared to those immunized intramuscularly. ID immunized mice showed decreased ear thickness as well as parasite burden compared to IM mice. This control that we observed was not quite as strong as mice that were previously infected with parasites (gold standard), suggesting there is room for improvement in our vaccine strategy.

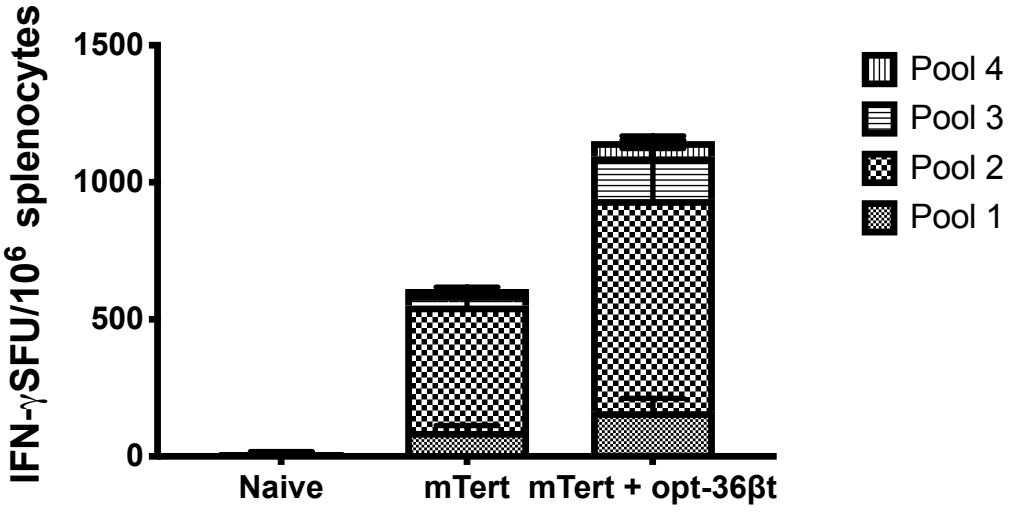


Figure 34. Opt36βt boosts IFN-γ T cell responses compared to vaccine alone against cancer antigen

C57BL/6 mice (n= 5 mice/group) were immunized three times, at two week intervals with either 25 µg of mTert vaccine alone, or 25 µg of mTert and 11 µg of opt36βt. Spleens were harvested 8 days after final immunization. ELISpot analysis of IFN-γ T cell responses depicted above.

Future directions: intradermal delivery

To date, the only human disease that has been globally eradicated is smallpox. Of note, this is one of the few vaccines, besides Bacille Calmette-Guerin (BCG) and rabies, currently administered intradermally. Work by Liu et al. (Liu et al. 2010) has shown that a major part of the success of this vaccine is due to skin delivery, not just the immunogenicity of the vaccine. As the skin is such an accessible tissue for vaccination, it would be reasonable to measure a vaccine's efficacy post intradermal delivery as well as the standard intramuscular delivery. The work highlighted in this thesis as well as clinical data generated in intradermal Zika and Ebola SDNA vaccine models especially highlight advantages in protective immune responses in the skin for infections transmitted through the skin by mosquitos and sandflies.

Future directions: PEPCK delivery

Given that we observed higher systemic responses with single immunization of IDM2 or PB at a 20 μg dose than we did when we administered both together in one vaccine at a 40 μg dose, we suspect that some antigenic interference is occurring. While it would be simpler and more clinically relevant to maintain the combination vaccination, future studies should be done to learn whether the immune responses elicited would be higher if the two plasmids were given at separate sites and if this affects the responses seen in the skin and lead to better parasite control.

While it has been difficult to estimate the number of Leishmania parasites transmitted during a sandfly bite, the best estimates put this number in the range of about 1000 parasites when sandfly transmission to mice is studied, which is much lower than the 2 million *L. major* parasites we delivered in our studies (M. E. Rogers et al. 2004). While there will be variability in people when infected with Leishmania parasites, revisiting our studies with a lower initial infectious dose

of parasite may better recapitulate what may happen in natural settings, and may allow us a better understanding of how efficacious the intradermal delivery of PEPCK can be.

All of the studies for intradermal DNA delivery illustrated in this thesis have been done in the C57Bl/6 mouse strain, as they represent the classic model to study *L. major* infection. C57BL/6 mice are known to generate immune responses that skew towards a Th1 response, which is needed for parasite and disease control. Th2 responses have long been associated with exacerbated disease, though a few reports show either no impact of Th2 antibody responses (Shahi et al. 2013; K. A. Rogers et al. 2002), or some protection. Balb/C mice, which are known to skew towards a Th2 response, generally develop severe ulcerating lesions and progress towards death following Leishmania challenge. Investigating whether PEPCK intradermal delivery in the skin can protect these mice from death and severe disease in spite of their genetic background would further support the case for the efficacy of vaccine delivery in the skin in the context of Leishmania.

Summary and concluding thoughts

In summary, we described through multiple studies the importance of the skin as a provider of cytokines that may be used to enhance vaccine responses as well as a privileged site that may offer a greater payoff in vaccine strategies. We show that intradermal delivery of PEPCK offers better disease control and parasite burden compared to intramuscular delivery after Leishmania challenge. We also show that truncated IL-36 gamma is able to enhance cellular responses post vaccination in three separate models and that it can provide significant protection against Zika challenge. Future studies should focus on developing and optimizing intradermal strategies across a number of infectious disease targets and cancer, as well as discovering novel molecules

that can serve as adjuvants to boost responses to vaccines that may not be immunogenic enough on their own.

The potential of the new synthetic DNA vaccine platform to radically improve global health cannot be understated. With the improvements in the platform, DNA represents an opportunity to rapidly respond to pandemics, expand therapies in resource strained settings that have few options, as well as provide clinical benefit to underserved populations that may have little recourse for treatment. From an economic standpoint, DNA vaccines require no viral or protein purification, no massive warehouses and tanks to generate product, allowing for greater manufacturing and accessibility to a wider audience. As more clinical studies are done to evaluate the efficacy of intradermal DNA delivery as well as the impact of localized delivery of gene adjuvants, skin delivery may play an important role in the development of this next generation of DNA vaccines (Figure A2).

The synthetic DNA platform has enjoyed a major boost in human potency due to a number of advancements, including electroporation, new DNA formulations, as well as genetic sequence optimization. The next generation of SDNA vaccines for remaining disease targets will likely also require multiple efforts to generate vaccine-induced protection. Preliminary data that I've generated suggests that codelivery of opt36βt, with IDM2 vaccine in an intramuscular vaccination site can enhance IFN-γ T cell responses (Figure 35). Opt36βt is particularly attractive as an adjuvant for PEPCK as it can enhance IFN-γ $CD4^+$ T cells, which is critical for protection in this model. Further studies looking at this vaccine formulation in an intradermal vaccination scheme will be critical to understanding whether there may be enhanced protection against Leishmania parasite challenge.

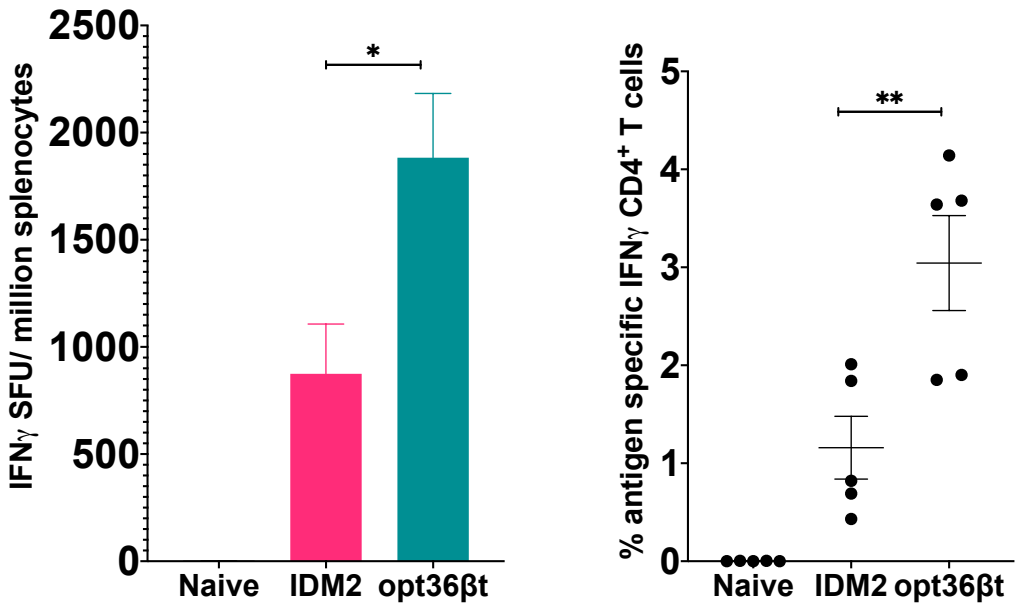


Figure 35. Codelivery of opt36βt with IDM2 enhances IFN-γ T cell responses in the spleen

C57BL/6 mice (n= 5 mice/group) were immunized two times, at two week intervals with either 20 µg of IDM2 vaccine alone, or 25 µg of IDM2 and 11 µg of opt36βt. Spleens were harvested 10 days after final immunization. ELISpot analysis of IFN-γ T cell responses depicted above.

CHAPTER 6- Appendix

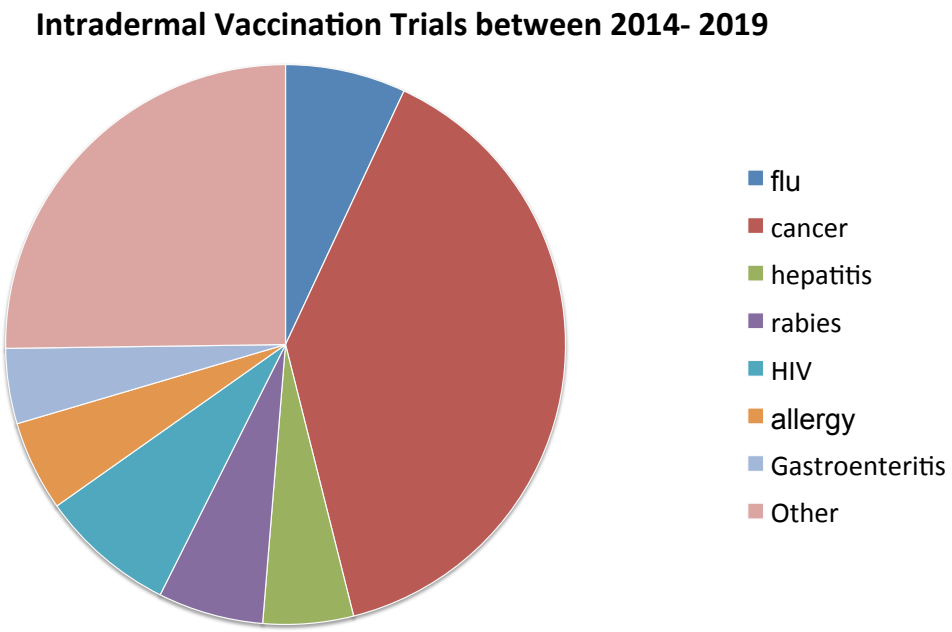


Figure A 1. Breakdown of intradermal vaccination clinical targets between Jan. 1, 2014 and Oct. 31, 2019

Clinical trials launched within the past five years are testing intradermal vaccination against a wide range of disease targets and conditions

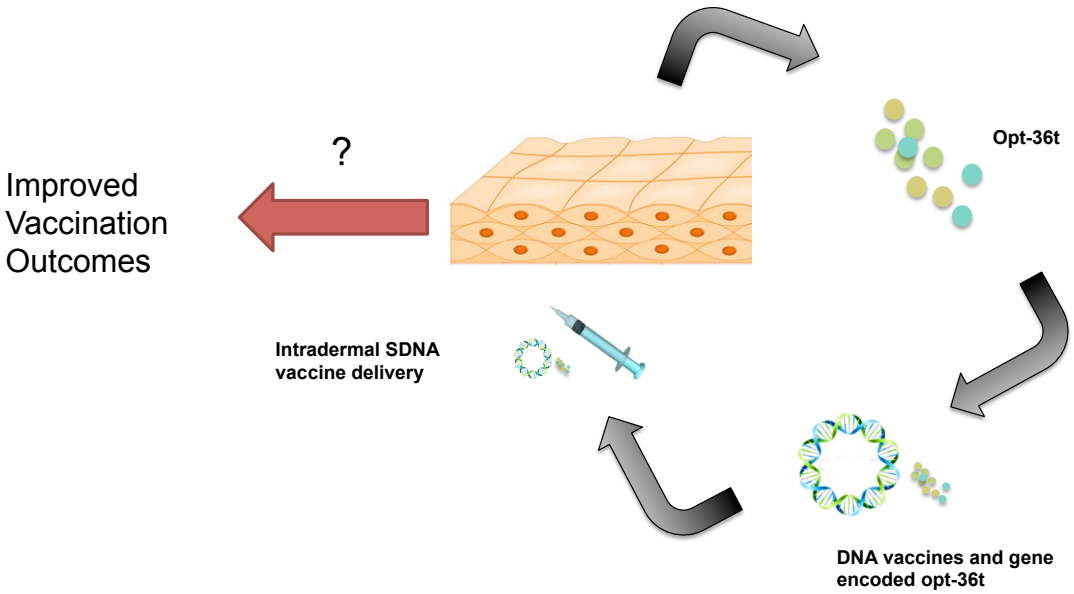


Figure A 2. Skin derived IL-36 cytokines in combination with skin vaccination may drive enhanced immune responses against SDNA vaccines

CHAPTER 7- Publications

The following papers have been published in support of this dissertation, starting with the most recently published work.

Intradermal synDNA vaccination generates Leishmania specific T cells in the skin and protection against *Leishmania major*.

Lumena Louis, Megan Clark, Megan C Wise, Nelson Glennie, Andrea Wong, Kate Broderick, Jude Uzonna, David B Weiner, Phillip Scott

Abstract

Vaccination remains one of the greatest medical breakthroughs in human history, and has resulted in near eradication of many former lethal diseases in many countries including the complete eradication of smallpox. However, there remain a number of diseases for which there are no or only partially effective vaccines. There are numerous hurdles in vaccine development, of which knowing the appropriate immune response to target is one of them. Recently, tissue resident T cells have been shown to mediate high levels of protection for several infections, although the best ways to induce these cells is still unclear. Here we compare the ability to generate skin resident T cells in sites distant from the immunization site following intramuscular and intradermal injection using optimized synthetic DNA vaccines. We found that mice immunized intradermally with a synthetic consensus DNA HIV Envelope vaccine by electroporation (EP) are better able to maintain durable antigen specific cellular responses in the skin compared to mice immunized by the intramuscular route. We extended these studies by delivering a synDNA vaccine encoding *Leishmania* Glycosomal Phosphoenolpyruvate Carboxykinase (PEPCK) by EP, and again found that the intradermal route was superior to the intramuscular route for generating skin resident PEPCK specific T cells. When challenged with *Leishmania major (L. major)*

parasites, we observed that mice immunized intradermally exhibited significant protection, while mice immunized intramuscularly did not. The protection seen in intradermally vaccinated mice supports the viability of this platform to not only generate skin resident T cells, but also to promote durable protective immune responses at relevant tissues sites.

Designed DNA-Encoded IL-36 Gamma Acts as a Potent Molecular Adjuvant Enhancing Zika Synthetic DNA Vaccine Induced Immunity and Protection In a Lethal Challenge Model

Lumena Louis, Megan C. Wise, Hyeree Choi, Daniel O. Villarreal, Kar Muthumani, David B. Weiner

Abstract

Identification of novel molecular adjuvants which can boost and enhance vaccine-mediated immunity and provide dose sparing potential against complex infectious diseases and for immunotherapy of cancer is likely to play a critical role in the next generation of vaccines. Given the number of challenging targets for which no or only partial vaccine options exist, adjuvants that can address some of these concerns are in high demand. Here, we report that a designed truncated IL-36 gamma encoded plasmid can act as a potent adjuvant for several DNA encoded vaccine targets including HIV, influenza, and Zika in immunization models. We further show that the truncated IL-36 gamma (opt-36gt) plasmid provides improved dose sparing as it boosts immunity to a suboptimal dose of a Zika DNA vaccine resulting in potent protection against a lethal Zika challenge.

Rapid Synthetic DNA vaccine development for emerging infectious disease outbreaks

Lumena Louis and David B. Weiner

Abstract

Vaccines are considered among the top feats of modern medicine, saving millions of lives by inducing immunity to a number of infectious pathogens. As the next generation of vaccines seeks to address ever more complicated targets including cancer, innovative technologies like synthetic DNA vaccination that circumvent some of the issues associated with traditional vaccines will likely prove critical. In addition, compounding factors that may influence immune outcome such as the microbiome must also be studied in greater detail. Recent clinical studies have suggested that the presence of certain bacteria in the gut was associated with favorable outcomes in patients receiving immunogenic chemotherapy. Other studies have also shown that a dysbiosis or overrepresentation of other bacteria strains was negatively associated with favorable outcome. Further work needs to be done to more fully understand the influence that the microbiome exerts on the immune system and vice versa, and the significance of this relationship in designing future therapies.

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